SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 14: 3065-3070, 2017

Methylation of *STK11* promoter is a risk factor for tumor stage and survival in clear cell renal cell carcinoma

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Received December 1, 2015; Accepted May 16, 2017

DOI: 10.3892/ol.2017.6534

Abstract. Inactivation of tumor suppressor gene serine-threonine kinase 11 (*STK11*) in clear cell renal cell carcinoma (ccRCC) has been demonstrated; however, the mechanism of this inactivation remains to be investigated. To investigate whether epigenetic alteration plays a role in the inactivation of *STK11* in RCC, the present study aimed to investigate the methylation status of the *STK11* promoter and its association with tumor stage and survival in ccRCC patients. Paraffin-embedded specimens were obtained from 42 ccRCC patients. The specimens were analyzed for the methylation status of the *STK11* promoter CpG island using methylation-specific polymerase chain reaction. Survival, tumor-node-metastasis (TNM)/American Joint Committee on Cancer (AJCC) stages, and hematological parameters were compared between patients with unmethylated (U), partially methylated (P) and methylated (M) *STK11* promoter. Among the 42 patients, there were 12 (28.6%), 18 (42.9%) and 12 (28.6%) patients in the M, P and U groups, respectively. The methylation status of the *STK11* promoter was associated with T, N and AJCC stages in RCC. Survival analysis showed that the M group had a significantly shorter survival time compared with the P and U groups. These findings suggested that methylation of the *STK11* promoter in RCC is a not rare event, and it may have an important role in the pathogenesis of RCC and be a risk factor for the prognosis of RCC.

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Abbreviations: RCC, renal cell carcinoma; ccRCC, clear cell RCC; STK11, serine-threonine kinase 11; LKB1, liver kinase B1; TNM, tumor-node-metastasis; AJCC, American Joint Committee on Cancer; MSP, methylation-specific polymerase chain reaction

Key words: clear cell renal cell carcinoma, promoter methylation, *STK11*

Introduction

Renal cell carcinoma (RCC) is the most common primary renal malignancy, and accounts for 2-3% of adult malignancies (1). In China, the incidence of RCC is ~540 cases per million individuals every year (2). It has been shown that RCC has a high mortality rate, and the 5-year survival rate of metastatic RCC patients is <10% (3). RCC can be divided into several subtypes according to the morphological and microscopic features, and clear cell RCC (ccRCC) is the most predominant subtype, which accounts for 75-80% of all RCCs (4).

The tumor suppressor serine-threonine kinase 11 (*STK11*), also termed liver kinase B1 (*LKB1*), was first identified as a germline-mutated gene in Peutz-Jeghers Syndrome in 1996 (5). The product of the *STK11* gene is a 50-kDa serine-threonine kinase involved in various biological functions, including cell polarity, cell detachment and adhesion, cell structure and energy metabolism (6). Germline mutations of the *STK11* gene are found in a variety of cancer types, including lung cancer (7), hepatocellular carcinoma (8) and breast cancer (9). In addition, functional studies showed that *STK11* heterozygous knockout mice would develop tumors in several organs (10,11).

Somatic mutations of the STK11 gene have also been found in several tumors, including pancreatic cancer (12), biliary cancer (12), hepatocellular carcinoma (8) and testicular tumor (13); however, the frequency of mutations is relatively rare, with a range of 0-6%. In lung cancer, a geographically variable incidence was observed. Mutational inactivation of the *STK11* gene is frequently detected in Caucasian, but not in Asian, lung cancer patients (14). As for RCC, a study by Avizienyte *et al* (15) detected no somatic mutations in the *STK11* gene in 19 RCC specimens, whereas a controversial result was observed by Yalniz *et al* (16), in which 51.6% of RCC patients were found to have somatic mutations in the *STK11* gene. Decreased expression of *STK11* has also been shown to occur in several cancer types, such as non-small cell lung cancer (8), breast carcinoma (17) and ccRCC. Duivenvoorden *et al* (18) showed that under-expression of *STK11* was a common event in all 10 examined ccRCC samples. However, the mechanism of reduced expression of *STK11* in ccRCC remains to be elucidated.

Although inactivation of *STK11* gene was found in several cancers, its somatic mutations appear rare. This indicates

that the under-expression of the *STK11* gene may be also mediated by other mechanisms. In addition to mutation, the expression of *STK11* can also be regulated through epigenetic modification, transcriptional regulation and post-translational modification (19). Epigenetic alterations that suppress the activity of tumor suppressor genes is an alternative mechanism for tumor development and progression (20). The methylation status of the *STK11* promoter has been investigated in colorectal cancer (21), non-small cell lung cancers (22), and breast, gastric, pancreatic, thyroid, bladder and testicular carcinomas (23). These studies reported that frequency of hypermethylation of the *STK11* promoter in the described tumors is low (0-13%) (21-23); however, this indicates that *STK11* promoter methylation contributes to the inactivation of the *STK11* gene and *STK11*-mediated functions.

At present, the methylation status of the *STK11* promoter in RCC cells remains unclear. In addition, the role of the methylation status of the *STK11* promoter in the pathogenesis of RCC remains to be investigated. In order to determine the possible inactivation of *STK11* by epigenetic mechanisms, the present study aimed to investigate the methylation status of the *STK11* promoter in ccRCC and its association with tumor disease stage and survival of ccRCC patients. The methylation status of the *STK11* promoter in RCC was determined by analysis of 42 cases of ccRCC Paraffin-embedded specimens were assessed using methylation-specific polymerase chain reaction (MSP) and the association with RCC progression was analyzed.

Patients and methods

Patients. The present study was reviewed and approved by the Institutional Review Board of the First Affiliated Hospital of Sun Yat-sen University (Guangzhou, China). Paraffin-embedded tumor specimens were obtained and prepared from 42 patients with ccRCC (29 men and 13 women) admitted to the First Affiliated Hospital of Sun Yat-sen University between February 1999 and August 2009. Patients pathologically diagnosed with ccRCC were included, and patients that did not comply with follow-up visits were excluded from the study. Clinical data, including the tumor-node-metastasis (TNM)/American Joint Committee on Cancer (AJCC) staging (9), hematological parameters and post-operative follow-up were documented for further analyses. Written informed consent was obtained from all patients prior to inclusion in the present study.

MSP. A total of 42 paraffin-embedded tissues were sectioned into 5 μm thick slices, then dried for 2 h at 60˚C or overnight at 37˚C. The slices were immersed in 2X xylene for 15 min, and then dehydrated through a graded series of ethanol (70, 80, 90 and 95%; 5 min each). DNA from the tissue samples was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacture's protocol. EZ DNA Methylation kit (Zymo Research Corp., Irvine, CA, USA) was used for bisulfite conversion to assess the DNA methylation status. The bisulfite-modified DNA was then used as a template, together with primers specific for methylated and unmethylated sequences for MSP. Polymerase chain reaction (PCR) was performed with DNA polymerase (Beijing Sunbiotech, Beijing, China) at a final volume of 25 μl. The primers used are as previously reported (10): Primers specific for methylated sequence were *STK11* forward 5'-ACGAAGTTG ATTTTGATCGGGTC-3' and reverse 5'-CGATACAAAATC TACGAACCGACG-3', whereas those for the unmethylated sequence were *STK11* forward, 5'-GGATGAAGTTGATTT TGATTGGGTT-3', and reverse, 5'-ACCCAATACAAA ATCTACAAACCAACA-3'; *GAPDH* forward 5'-GGAGCG AGATCCCTCCAAAAT-3' and reverse 5'-GGCTGTTGT CATACTTCTCATGG-3'. All primers were synthesized and purchased from Zymo Research (USA). PCR fragments were 122 bp in length. The reaction consisted of initialization at 95˚C for 10 min, followed by 35 cycles of denaturation at 95˚C for 30 sec, cooling at 57˚C for 59 sec and extension at 72˚C for 30 sec, and final extension at 72˚C for 10 min. The PCR products were analyzed on a 1% agarose gel (Beijing Hengao Biotechnology, Beijing, China); DNA bands were captured using a UV gel imaging system (EC3 Imaging system, UVP LLC, Upland, CA, USA). The presence of methylated and unmethylated bands in the PCR product indicated partial methylation, the presence of only a methylated band indicated methylation, and the presence of only an unmethylated band indicated unmethylation.

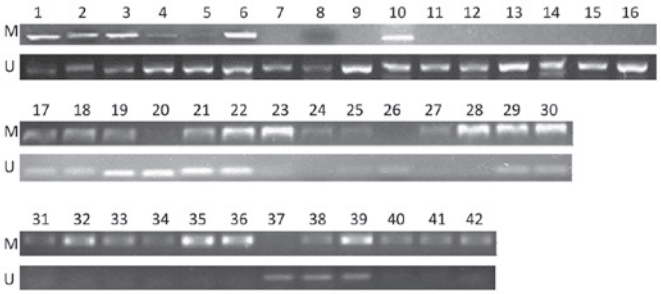

Figure 1. Methylation status of serine-threonine kinase 11 in clear cell renal cell carcinoma tissues was determined by methylation-specific polymerase chain reaction. 1-42 indicates the patient number. U, unmethylated, M, methylated.

Statistical analysis. Statistical analysis was performed using R 3.0.2. All data are presented as the mean ± standard deviation. Significance was assessed using analysis of variance followed by Tukey Honestly Significant Difference test for all baseline characteristics and hematological parameters, with the exception of the TNM stage, AJCC stage, blood type and sex, which were analyzed using Fisher's exact test. Survival curves of patients in the three groups were plotted and the differences between the three curves were estimated by log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Methylation status of STK11 promoter in ccRCC. The methylation status of the *STK11* promoter in 42 ccRCC paraffin-embedded tissue samples was determined using MSP. The data showed that, among the 42 samples, there were 12 (28.6%), 18 (42.9%) and 12 (28.6%) samples in the methylation group (M group), partial methylation group (P group) and unmethylation group (U group), respectively, based on the status of the *STK11* promoter (Fig. 1).

Table I. Demographic data of clear cell renal cell carcinoma patients in the M, P and U groups with regard to serine-threonine kinase 11 promoter status (n=42).

Characteristic	Group			P-value
	M	P	U	
Total, n	12	18	12	
Sex, n (%)				0.1783
Male	10 (83.3)	9 (50.0)	9 (75.0)	
Female	2 (16.7)	9 (50.0)	3 (25.0)	
Age, years (SD, range)	50.8 (13.4, 33-82)	44.4 (15.5, 14-68)	49.9 (12.9, 27-67)	0.413
Height, cm (SD, range)	165.1 (8.7, 152-176)	160.2 (9.5, 150-178)	163.8 (7.8, 147-171)	0.292
Body weight, kg (SD, range)	62.4 (10.6, 47-86.5)	59.4 (13.8, 41-85)	65.2 (12.6, 47-82)	0.474
BMI, kg/m^2 (SD, range)	22.7 (2.2, 20-28)	23.0 (4.2, 17-31)	24.2 (3.6, 17-29)	0.565
Tumor diameter, cm (SD, range)	6.9 (2.98, 3.5-13)	6.7 (2.74, 2.5-11)	6.71 (2.71, 2.9-11.5)	0.981
Follow-up time, months (SD, range)	47.17 (22.64, 20-94)	95.25 (51.13, 14-153)	92.56 (45.53, 19-150)	0.010

SD, standard deviation; M, methylated; P, partially methylated; U, unmethylated; BMI, body mass index.

Table II. Hematological parameters of clear cell renal cell carcinoma patients in the M, P and U groups with regard to serine-threonine kinase 11 promoter status (n=42).

Parameter	Group			P-value
	M	P	U	
K^+, mmol/l (SD, range)	4.43 (0.64, 3.7-5.9)	4.15 (0.39, 3.5-4.9)	4.43 (0.34, 3.9-4.9)	0.382
Na^+, mmol/l (SD, range)	140.1 (2.9, 136-145)	139.9 (2.3, 135-142)	141.7 (5.5, 131-149)	0.554
Cl^+, mmol/l (SD, range)	105.5 (5.1, 96-115)	103.9 (4.1, 99-114)	105.6 (7.8, 90-114)	0.772
$Ca2^+$, mmol/l (SD, range)	2.31 (0.47, 1.03-2.91)	2.38 (0.12, 2.09-2.55)	2.12 (0.46, 1.02-2.5)	0.219
AST, U/l (SD, range)	23.6 (13.5, 13-57.2)	22.1 (9.4, 6-41)	22.8 (12.1, 8-51)	0.934
ALT, U/l (SD, range)	28.1 (25.9, 6-89.9)	21.1 (14.2, 7-56)	23.4 (12.4, 3-41)	0.584
TBA, mmol/l (SD, range)	4.0 (1.9, 1.1-7.1)	5.7 (2.9, 2.2-11.5)	6.6 (5.1, 1.7-20.8)	0.226
ALP, U/l (SD, range)	73.1 (21.1, 42-117.1)	67.5 (35.2, 35-190)	73.8 (27.8, 35-136)	0.811
GGT, U/l (SD, range)	36.2 (33.4, 10-132)	30.6 (26.5, 2.4-93)	37.8 (31.8, 1-99)	0.787
LDH, U/l (SD, range)	156 (31.3, 114-208)	202 (48.8, 133-296.3)	187 (91.8, 82-346)	0.142
AFU, nmol/ml·h (SD, range)	12.9 (5.97, 5-26)	10.2 (4.92, 5-21)	8.78 (5.53, 2-19.4)	0.191
ALB, g/l (SD, range)	40.3 (3.99, 34.6-47.4)	43.1 (3.6, 37.5-48.6)	40.5 (5.16, 30.3-48.6)	0.116
GLO, g/l (SD, range)	28.1 (5.9, 22.1-38)	31.5 (4.72, 22.2-40.8)	30.7 (5.75, 22.5-43.9)	0.223
DBIL, μmol/l (SD, range)	3.37 (2.39, 1.1-10.1)	3.34 (1.88, 0.22-8.87)	3.37 (2.23, 0.76-8.94)	0.999
IBIL, μmol/l (SD, range)	7.27 (3.02, 3.1-11.72)	11.5 (8.6, 3.4-40.3)	10.7 (5.4, 0.81-20.83)	0.249
BUN, mmol/l (SD, range)	5.03 (2.53, 2.13-10.11)	5.1 (1.61, 2.82-8.01)	5.11 (1.71, 2.49-8)	0.994
CRE, μmol/l (SD, range)	99.3 (26.1, 67.29-141)	92.5 (21.1, 51.3-134)	93.2 (28.7, 29-133)	0.747
UA, μmol/l (SD, range)	326 (89.8, 172.6-454)	323 (94.1, 118-551)	375 (144.5, 87-597)	0.403
CHO, mmol/l (SD, range)	4.34 (0.97, 2.97-5.4)	4.91 (1.10, 3.61-7.52)	4.39 (0.85, 3.14-6)	0.233
TG, mmol/l (SD, range)	1.15 (0.62, 0.54-2.51)	1.52 (0.92, 0.53-3.96)	2.02 (1.42, 0.65-5.19)	0.126
GLU, mmol/l (SD, range)	4.92 (0.77, 3.22-5.94)	5.08 (0.73, 4.16-6.89)	5.07 (0.58, 3.68-5.96)	0.819
HDL, mmol/l (SD, range)	1.17 (0.42, 0.59-2.26)	1.25 (0.33, 0.82-1.87)	1.09 (0.22, 0.71-1.47)	0.413
LDL, mmol/l (SD, range)	2.67 (0.82, 1.47-4.12)	3.0 (0.83, 1.46-4.53)	2.72 (0.93, 1.64-4.46)	0.532
WBC, 10^9/l (SD, range)	8.04 (1.92, 4.6-11)	8.11 (2.52, 5.1-13.5)	8.73 (2.44, 5-14)	0.725
RBC, 10^{12}/l (SD, range)	4.64 (0.61, 3.35-5.71)	4.48 (0.99, 2.79-6.76)	4.77 (0.72, 3.79-6.32)	0.647
Hb, g/l (SD, range)	132 (17.4, 95.3-156)	129 (25.3, 79-165)	135 (21.8, 93-171)	0.750
PLT, 10^9/l (SD, range)	285 (91.6, 179-475)	272 (124.6, 128-553)	235 (33.8, 198-286)	0.420

SD, standard deviation; M, methylated; P, partially methylated; U, unmethylated.

Patient demographic data. To investigate the effect of the methylation status of the *STK11* promoter on ccRCC, the 42 enrolled patients were grouped into the M, P and U groups, according to the methylation status of the *STK11* promoter. The patient demographic data of the 3 groups is shown in Table I. In general, with the exception of the follow-up time, there were no significant differences in the clinical characteristics among the 3 groups. Comparison of hematological parameters among the three groups also showed no significant difference (Table II). These results revealed the equivalence of demographic and clinical characteristics of the three groups.

Association between STK11 promoter methylation and TNM/AJCC staging. To investigate whether the *STK11* promoter methylation status is associated with the disease stage of RCC, the distributions of TNM and AJCC stages among the three groups were investigated. As shown in Table III, all stage distributions were significantly different between the 3 groups. There was a statistically significant difference in the distribution of the T (P=0.036), N (P=0.007) and AJCC (P<0.001) stages among the M, P, and U groups. In addition, significant or marginally significant trends were observed that the M group had more patients with advanced stage disease than the P and U groups (P<0.10 for T and N stages, P<0.05 for M and AJCC stages; residual analysis). The data suggested that the methylation status of the *STK11* promoter was associated with T, N and AJCC stages in RCC.

STK11 promoter methylation and survival. Since the association between methylation status and tumor stage was observed, whether the methylation status has an effect on the survival of RCC patients was then investigated. The results of Kaplan-Meier survival analysis showed that there was a significant survival difference among the three groups (log-rank test, P<0.05; Fig. 2A). Additional analysis revealed that the survival times of patients in the P (P=0.021) and U (P=0.048) groups were significantly increased compared with the M group (Fig. 2B and C). However, there was no significant difference in survival time between the U and P groups (P=0.640; Fig. 2D). The data suggest that the methylation status of the *STK11* promoter has an impact on the survival of RCC patients.

Table III. TNM and AJCC staging based on the methylation status of the serine-threonine kinase 11 promoter (n=42).

	Group, n (%)			
Variable	M	P	U	P-value
Total, n	12	18	12	
T stage				0.036
T1	4 (33.3)	8 (44.4)	8 (66.7)	
T2	3 (25.0)	10 (55.6)	3 (25.0)	
T3	3 (25.0)	0 (0.0)	1 (8.3)	
T4	2 (16.7)	0 (0.0)	0 (0.0)	
N stage				0.007
N0	5 (41.7)	15 (83.3)	11 (91.0)	
N1	3 (25.0)	3 (16.7)	0 (0.0)	
N2	4 (33.3)	0 (0.0.)	1 (9.0)	
M stage				0.154
M0	9 (75.0)	17 (94.4)	12 (100.0)	
M1	3 (25.0)	1 (5.6)	0 (0.0)	
AJCC stage				<0.001
I	0 (0.0)	7 (38.9)	8 (66.7)	
II	3 (25.0)	9 (50.0)	3 (25.0)	
III	3 (25.0)	0 (0.0)	0 (0.0)	
IV	6 (50.0)	2 (11.1)	1 (8.3)	

Fisher's exact test showed methylation status was associated with T, N and AJCC stages. TNM, tumor-node-metastasis; AJCC, American Joint Committee on Cancer; M, methylated; P, partially methylated; U, unmethylated.

Discussion

In the present study, *STK11* promoter methylation was analyzed using specimens from 42 ccRCC patients and found an association between methylation status and cancer stage. The results showed that 28.6 and 42.9% of ccRCC samples had methylation and partial methylation at the *STK11* promoter, respectively. Additional analyses found the methylation status of the *STK11* promoter was associated with the T, N and AJCC stages in RCC. In addition, the M group had an increased number of patients at an advanced stage compared with the P and U groups. Furthermore, survival analyses among three groups showed that the survival time was significantly longer in both P and U groups compared with the in M group, indicating that the methylation status of the *STK11* promoter has an impact on the survival of RCC patients. To the best of our knowledge, the present study is the first to report the methylation frequency of the *STK11* promoter in ccRCC and its impact on the tumor stage and survival of ccRCC patients.

STK11 has multiple biological and physiological functions in cells, since knockout mice studies have shown that inactivation of *STK11* has severe consequences, including tumorigenesis. Although *STK11* was identified almost two decades ago (5), the studies focusing on its roles in the pathogenesis of RCC remain rare. In 1999, Avizienyte *et al* (15) detected no mutation in 19 RCC specimens. In 2014, Yalniz *et al* (16) reported an overall mutation frequency of 51.6% (32/62) in RCC patients. In 2013, Duivenvoorden *et al* (18) conducted a study to investigate the tumor suppressor function of *STK11* in ccRCC *in vitro* and *in vivo*. Knockdown of *STK11* in the ccRCC 786-O cell line increased the cell proliferation, invasion and vascular endothelial growth factor secretion. In addition, the growth of *STK11* knockdown cell xenografts was significantly increased compared with the control. These results suggested a tumor suppressor function of *STK11* in ccRCC. In addition, this study also investigated the expression of *STK11* at the mRNA and protein levels, as well as performing immunohistochemistry staining. It was found that under-expression of STK11 in ccRCC is a comment event. However, this study did not further investigate the mechanism for the under-expression of *STK11* in ccRCC (16).

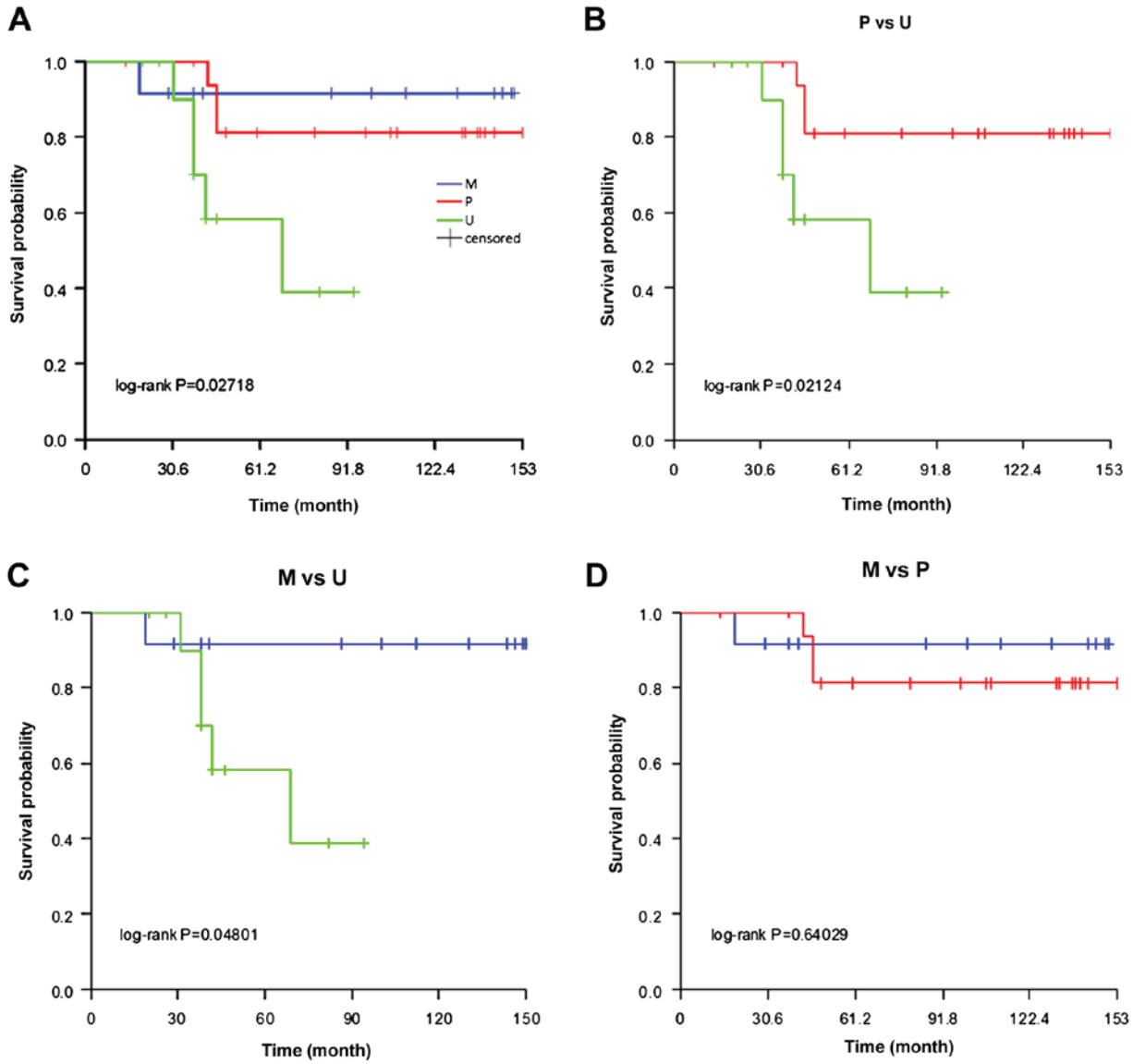

Figure 2. (A) Kaplan-Meier survival curves for overall survival in ccRCC patients with different STK11 promoter methylation status. The (B) P and (C) M groups had significantly better survival than the U group. (D) Survival in the M and P groups was not significantly different.

A previous study has already shown that mutation of the *STK11* gene may contribute to the inactivation of *STK11* (24). In the present study, to determine if epigenetic alteration may also contribute to inactivation of *STK11*, the methylation status of the *STK11* promoter region in 42 ccRCC specimens was investigated. Hypermethylation of the *STK11* promoter has been demonstrated in previous studies. In a cell line study, Esteller *et al* (23) showed that three colorectal and one cervical carcinoma cell lines were methylated at *STK11*. As for sporadic primary tumors, studies showed the methylation frequency of the *STK11* promoter in various tumors is rare. In the study by Esteller *et al* (23), a series of primary tumors were also investigated. Among colorectal, breast, gastric, pancreatic, thyroid, bladder and testicular carcinomas, only colorectal carcinoma (7.7%; 1/13) and testicular tumor (10.7%; 3/28) exhibited methylated at *STK11* (23). In another study by Trojan *et al* (21), an overall methylation frequency of 8% (4/48) was observed in colorectal cancer. Lee *et al* (22) reported that promoter methylation was detected in 13.2% (21/159) of Korean patients with non-small cell lung cancer. Notably, in contrast to these studies, the present study showed a significantly increased methylation rate (28.6%; 12/42) in patients with ccRCC. This finding may indicate that epigenetic alteration plays a more important role in the pathogenesis of RCC compared with other cancer types. However, whether this relatively high methylation frequency of *STK11* in ccRCC is a general phenomenon or may be attributed to the enrollment bias of the present study should be further verified in a subsequent study.

In the present study, the correlation between the methylation status of the *STK11* promoter and the tumor stage and survival of ccRCC patients was further analyzed. The results showed that the methylation status of the *STK11* promoter was associated with the tumor progress in RCC patients. Patients in the M group (with methylated at *STK11*) had a increased percentage of patients with advanced stages, using either the TNM or AJCC staging systems, compared with patients in the P and U groups. It is notable that the results of the survival analysis further support this observation. The survival time of

patients with methylated *STK11* (M group) was significantly lower than those in the U and P groups. The follow-up time of the M group was also significantly shorter than those of the U and P groups, which may be due to the fact that M group had a shorter survival time. These findings indicated that the methylation of *STK11* may be important in the pathogenesis of RCC and may be a risk factor for the prognosis of RCC. However, this conclusion should be further verified in a subsequent study with a large sample size to exclude the possibility of enrollment bias.

There are certain limitations in the present study. The expression level of mRNA and protein was not further investigated in these tumor samples to confirm the epigenetic inactivation of *STK11*. Secondly, the sample size of the present study was small. Thirdly, the surrounding normal tissues of the ccRCC tumor specimens were not simultaneously analyzed to identify the methylation difference in *STK11* between normal and tumor tissues. These limitations should be addressed in subsequent studies.

In summary, the present study investigated the methylation status of *STK11* and its association with tumor stage and survival of ccRCC patients. The methylation frequency of *STK11* was 28.4% in 42 ccRCC specimens. Patients in the M group had an increased percentage of patients with advanced stage RCC and a decreased survival time compared with the P and U groups. The present findings suggested the methylation status of *STK11* may be important in the tumorigenesis of ccRCC.

Acknowledgements

The present study was supported by the Science and Technology Foundation of Guangzhou (grant no. 2014A020212580).

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