Aberrant Promoter Methylation of *DLEC1*, a Critical 3p22 Tumor Suppressor for Renal Cell Carcinoma, is Associated With More Advanced Tumor Stage

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Purpose: Identifying tumor suppressor genes silenced by promoter CpG methylation uncovers mechanisms of tumorigenesis and identifies new epigenetic biomarkers for early cancer detection. *DLEC1* is located at 3p22.3, a critical tumor suppressor gene locus for renal cell carcinoma. We explored its epigenetic alteration in renal cell carcinoma and possible clinicopathological association.

Materials and Methods: We examined *DLEC1* expression and methylation by semiquantitative reverse transcriptase and methylation specific polymerase chain reaction in 9 renal cell carcinoma cell lines and 81 primary tumors. We also analyzed the relationship between *DLEC1* methylation and clinicopathological features in patients with renal cell carcinoma. We assessed *DLEC1* inhibition of renal cell carcinoma cell growth by colony formation assay.

Results: *DLEC1* methylation and down-regulation were detected in all renal cell carcinoma cell lines. Treatment with 5-aza-2′-deoxycytidine (Sigma®) and/or trichostatin A (Cayman Chemical, Ann Arbor, Michigan) reversed methylation and restored *DLEC1* expression, indicating that methylation directly mediates its silencing. Aberrant methylation was further detected in 25 of 81 primary tumors (31%) but only 1 of 53 nonmalignant renal tissues (2%) showed methylation. *DLEC1* methylation status was significantly associated with TNM classification and grade in patients with renal cell carcinoma (chi-square test $p = 0.01$ and 0.04, respectively). *DLEC1* ectopic expression in silenced renal cell carcinoma cells resulted in substantial tumor cell clonogenicity inhibition.

Conclusions: To our knowledge we report for the first time that *DLEC1* is often down-regulated by CpG methylation and shows tumor inhibitory function in renal cell carcinoma cells, indicating its role as a tumor suppressor. *DLEC1* tumor specific methylation may serve as a biomarker for early detection and prognosis prediction of this tumor.

Key Words: kidney; carcinoma, renal cell; DLEC1 (deleted in lung cancer) protein, human; methylation; genes, tumor suppressor

Abbreviations and Acronyms

AJCC = American Joint Committee on Cancer
Aza = 5-aza-2′-deoxycytidine
BGS = bisulfite genomic sequencing
CDH1 = *cadherin-1*
DLEC1 = deleted in lung and esophageal cancer
GAPDH = glyceraldehyde-3-dehydrogenase
LOH = loss of heterozygosity
MSP = methylation specific PCR
PCR = polymerase chain reaction
RASSF1A = *Ras association family 1A*
RCC = renal cell carcinoma
RT = reverse transcriptase
TSA = trichostatin A
TSG = tumor suppressor gene
VHL = *von Hippel-Lindau*

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RENAL cell carcinoma accounts for 3% of all adult malignancies and is the most common primary tumor arising from adult kidneys. Early stage RCC is usually asymptomatic but a significant percent of patients with RCC

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present with metastatic disease and experience low treatment efficacy. As reported previously, approximately 33% of patients with RCC have metastatic disease at presentation while metastasis eventually develops in 40% who undergo surgical resection.[1] Thus, novel, specific biomarkers are urgently needed for early detection of this tumor.

Elucidation of the molecular mechanisms underlying RCC development leads to the identification of promising targets for tumor early diagnosis and the further development of targeted therapy based on the biological pathways that are deregulated in an individual. Like other solid tumors, RCC arises by the accumulation of multiple genetic and epigenetic alterations.[2] Alterations at 3p, especially the 3p22-11 region, are among the most commonly detected tumor associated genetic changes in RCC cases.[3] Mutations in the *VHL* gene, often with LOH at 3p26-25, are the most common genetic events described in this cancer.[3] The LOH of *fragile histidine triad*, located at 3p14.2, was reported to be an early event in RCC development and a characteristic of all RCC types.[3]

As an alternative to genetic changes, DNA methylation of CpG rich promoter regions is now recognized as a major epigenetic mechanism to inactivate TSGs in cancer cases, which leads to transcription repressor binding, compressed chromatin and transcription silencing.[4] Tumor specific CpG methylation can provide clues to identify novel TSGs and biomarkers for early tumor detection and prognosis assessment. A growing number of aberrantly methylated TSGs have been reported in RCC, including *VHL; RASSF1A; p16; adenomatous polyposis coli; glutathione-S-transferase P; death associated protein kinase; CDH1; differentially expressed in adenocarcinoma of the lung; γ-catenin; serine peptidase inhibitor, Kunitz type, 2*; and *homeobox B13*.[5–10] Our group also recently reported frequent *deleted in liver cancer 1* methylation in RCC cases.[11] However, the rate of aberrant methylation of most TSGs is relatively low for RCC at about 10% to 30%. Thus, more studies are needed for RCC epigenetic alterations.

To identify critical TSGs and specific biomarkers for RCC, we studied 3p22-21.3, a chromosomal region with frequent LOH in various cancer types, including RCC.[12] Several candidate TSGs located at this locus, such as *RASSF1A; sema domain, Ig domain (Ig), short basic domain, secreted, (semaphorin) 3B; human ortholog of the yeast YA22; phospholipase C, δ 1*; and *BLU,* are inactivated by DNA methylation in tumors.[13] We recently identified *DLEC1*, located at the AP20 subregion, as a hypermethylated target with tumor suppressor functions in multiple tumors, including hepatocellular, colorectal, gastric, esophageal and nasopharyngeal carcinoma.[14,15] In the current series we explored its epigenetic alteration in RCC, and the relationship between *DLEC1* methylation and clinicopathological features in patients with RCC, which to our knowledge have not been previously studied. We also assessed its ability to inhibit RCC cell clonogenicity.

MATERIALS AND METHODS

Patients and Tissue Samples

All human primary RCCs (81 cases) and adjacent nonmalignant renal tissue (53) were obtained from the urology department, Peking University First Hospital, Beijing, People's Republic of China, from January 2005 to March 2006. Patients provided consent according to university policy. Table 1 lists patient clinicopathological features. The male-to-female ratio was 2.38:1 (57:24). Mean age overall was 52.0 years (range 21 to 76), and in patients with methylated and unmethylated *DLEC1* promoter it was 56.9 and 53.2 years, respectively ($p = 0.34$). All cases were collected from primary surgical resection with no prior history of RCC and adjuvant therapy. Specimens were snap frozen in liquid nitrogen and subsequently stored at −80C. Pathological diagnosis was done and confirmed at the pathology department, Institute of Urology, Peking University First Hospital. Tumors were histopathologically classified by 2002 AJCC TNM stage.[16]

Cell Lines and Drug Treatment

The RCC cell lines 786-O, CaKi-2, ACHN, A498, CaKi, HH050, HH244, RCC52 and RCC98, the human normal embryonic kidney cell line HEK293 transformed with sheared human adenovirus type 5 (HAd5) DNA and the nontumorigenic human kidney epidermal keratinocyte cell line RHEK-1 immortalized by Adl2-SV40 virus as controls were routinely maintained in RPMI1640 or Dulbecco's modified Eagle's medium with 10% fetal bovine

Table 1. *Clinicopathological features in patients with RCC and DLEC1 methylation status*

Clinicopathological Features	No. Methylated (%)	No. Unmethylated (%)	p Value
Overall	25 (30.9)	56 (69.1)	Not significant (Student's t test)
Gender:			
M	21 (36.8)	36 (63.2)	Not significant (Fisher's exact test)
F	4 (16.7)	20 (83.3)	0.113
Side:			
Rt	10 (28.6)	25 (71.4)	Not significant (Fisher's exact test)
Lt	15 (32.6)	31 (67.4)	0.180
TNM classification:			
pT1a	7 (17.5)	33 (82.5)	0.01 (chi-square test)
pT1b	5 (33.3)	10 (66.7)	
pT2	6 (42.9)	8 (57.1)	
pT3	7 (58.3)	5 (41.7)	
Nuclear grade:			
G1	3 (15.0)	17 (85.0)	0.04 (chi-square test)
G2	13 (29.5)	31 (70.5)	
G3	9 (52.9)	8 (47.1)	

serum, 100 U/ml penicillin G and 100 mg/ml streptomycin. Cells were incubated at 37C in a humidified atmosphere with 5% CO_2. Cell lines were treated with 10 μM Aza with or without 100 nM TSA, as described previously.[17]

DNA and RNA Extraction

DNA and RNA extraction from cell lines was done as described previously,[11,17] using TRI Reagent®. For DNA extraction from tissue the tumor and normal kidney tissues were homogenized in the presence of liquid nitrogen and incubated in 10 mM tris HCl (pH 8.0), 50 mM ethylenediaminetetraacetic acid, 10 mM NaCl, 2% N-lauryl sarcosyl and 200 μg/ml proteinase K for 20 hours at 55C, followed by phenol-chloroform extraction and ethanol precipitation.[11]

Semiquantitative RT-PCR

DLEC1 expression was determined by semiquantitative RT-PCR using the RNA PCR kit (Applied Biosystems®) and Go Taq®. Table 2 lists primer sequences and cycling parameters.

MSP and BGS

DNA bisulfite treatment, MSP and BGS were done according to our previous reports.[11,18] Table 2 lists MSP and BGS primers, and PCR conditions. MSP primers were tested previously for not amplifying any unbisulfited DNA and MSP products of several primary tumors were confirmed by direct sequencing with BigDye® v3.1, indicating that our MSP system was specific. Amplified BGS products were TA cloned and 5 to 10 colonies were randomly chosen and sequenced.

Colony Formation Analysis

Cells (1.5×10^5 per well) were plated in a 12-well plate and transfected with expression plasmid pcDNA3.1-DLEC1 or the empty vector pcDNA3.1 (0.8 μg each) using FuGene® 6. At 48 hours after transfection cells were collected and replated in a 6-well plate and selected for 2 weeks with G418 (0.4 mg/ml). Surviving colonies (50 or greater cells per colony) were counted after staining with gentian violet. Total RNA from transfected cells was extracted, treated with TURBO DNase™ and analyzed by RT-PCR to confirm *DLEC1* ectopic expression. All experiments were done 3 times in triplicate wells.

Table 2. *Primers*

PCR (primer)	Sequence	Product Size (bp)	No. PCR Cycles	Annealing Temperature (C)
RT-PCR				
DLEC1A	ttcctccctcgcctactc	309	35	55
DLEC1B	aaactcatccagccgctg			
MSP:				
DLEC1m1	gtttcgtagttcggtttcgtc	107	40	58
DLEC1m2	cgaaatatcttaaatacgcaacg			
DLEC1u1	tagttttgtagtttggttttgtt	110	40	55
DLEC1u2	acaaaatatcttaaatacacaaca			
BGS:				
DLEC1BGS56	gggtttagtagttttagttag	429	40	58
DLEC1BGS4	caactacaaccccaaatcctaa			

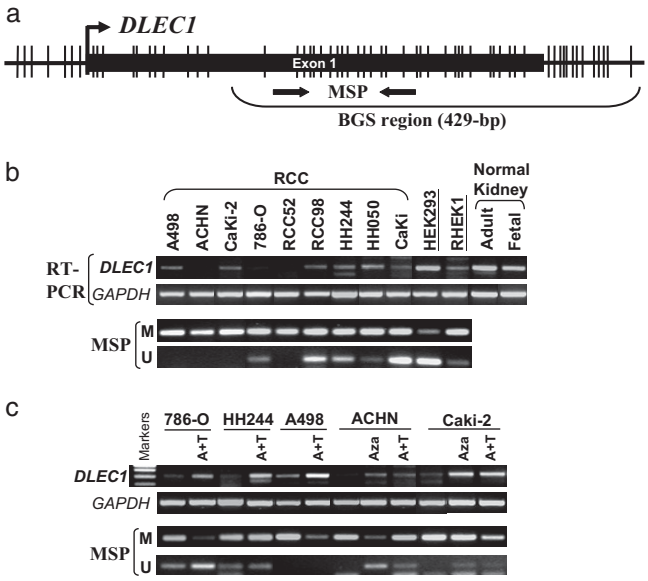

Figure 1. *DLEC1* down-regulation by promoter methylation in RCC cell lines. *a*, *DLEC1* promoter CpG island, with exon 1, CpG sites (vertical lines), MSP and BGS regions. Arrow indicates transcription start site. *b*, *DLEC1* expression and methylation in RCC, HEK293 and RHEK-1 cell lines on RT-PCR and MSP with *GAPDH* as control. *DLEC1* was readily detected in normal fetal and adult renal tissues. *M*, methylated. *U*, unmethylated. *c*, pharmacological demethylation using Aza with or without TSA induced *DLEC1* expression in methylated and silenced cell lines. *A+T*, Aza and TSA.

Statistical Analysis

Experimental differences were tested for statistical significance using the 2-tailed t-test, Fisher exact test or chi-square test with $p < 0.05$ considered significant.

RESULTS

DLEC1 in RCC Cell Lines

Down-regulation due to promoter CpG methylation. We first examined the *DLEC1* expression in 9 RCC cell lines by semiquantitative RT-PCR. Results showed that *DLEC1* was silenced or down-regulated in all cell lines compared to its expression in normal fetal and adult renal tissues (fig. 1, *b*). HEK293 cells also showed high *DLEC1* expression while the immortalized RHEK-1 cell line had only weak expression. The region spanning the putative promoter and exon 1 of *DLEC1* is a typical CpG island and, thus, susceptible to epigenetic silencing (fig. 1, *a*). We designed MSP and BGS primers to analyze its methylation status. On MSP *DLEC1* methylation was detected in all RCC cell lines and RHEK-1 but only weak methylation was detected in HEK293 (fig. 1, *b*). Further detailed methylation analysis using BGS in 3 RCC cell lines confirmed MSP results with high density of methylated CpG sites detected in all (fig. 2). Results showed that *DLEC1* down-regula-

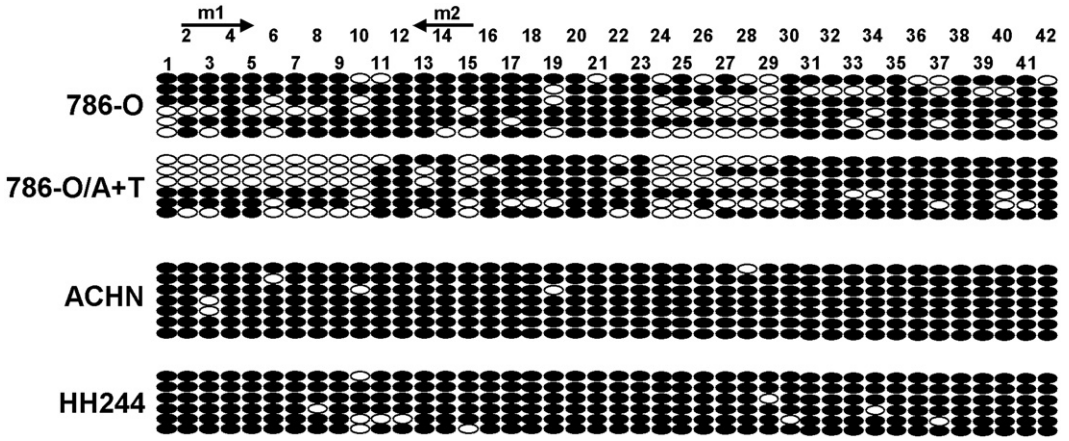

Figure 2. BGS high resolution mapping of methylation status of each CpG site (ovals) in *DLEC1* promoter. Rows represent individual allele of *DLEC1* promoter analyzed. Arrows indicate MSP primer sites. Filled ovals indicate methylated. Open ovals indicate unmethylated. *A+T*, Aza and TSA.

tion is common in RCC cell lines and closely correlates with its promoter methylation.

Expression restoration after demethylation agent treatment. To determine whether methylation directly mediates *DLEC1* silencing the 5 cell lines 786-O, A498, ACHN, HH244 and CaKi-2 were treated with the DNA methyltransferase inhibitor Aza with or without the histone deacetylase inhibitor TSA. After treatment *DLEC1* expression was significantly increased in these cell lines along with an increase in unmethylated promoter alleles and a decrease in methylated alleles (fig. 1, *c*). Further detailed BGS methylation analysis for 786-O before and after Aza treatment confirmed its demethylation (fig. 2). Results showed that CpG methylation of the *DLEC1* promoter directly led to its silencing in RCC.

Ectopic Expression of *DLEC1* Inhibited Renal Tumor Cell Clonogenicity

To examine the potential tumor suppressor functions of *DLEC1* in RCC cells *DLEC1* expressing construct was transiently transfected into 786-O and HH244 cells with methylated and silenced *DLEC1*, and assessed with anchorage dependent colony formation assays. After selecting transfected cells with G418 for 2 weeks the number of colonies formed in *DLEC1* transfectants was significantly less than that in empty vector transfectants, ie down to 21% in 786-O and 16% in HH244, indicating that DLEC1 strongly suppressed RCC cell growth (fig. 3).

DLEC1 Methylation and RCC Clinicopathological Features

We further analyzed 81 primary RCC samples and 53 tumor adjacent, nonmalignant renal tissues from the same patients for *DLEC1* methylation status and clinicopathological features (table 1 and fig. 4). Of 81 tumor samples 25 (31%) showed methylation while only 1 of 53 normal renal tissues (2%) had methylation. Unmethylated bands were detected in all RCC and all nonmalignant samples. Figure 4 shows representative MSP results, which were confirmed by direct sequencing of methylation specific and unmethylation specific bands in some cases (data not shown). Further detailed methylation analysis using BGS in 6 RCC and 2 normal tissues confirmed MSP results. In RCC-C22, C26, C81, C49 and C57, in

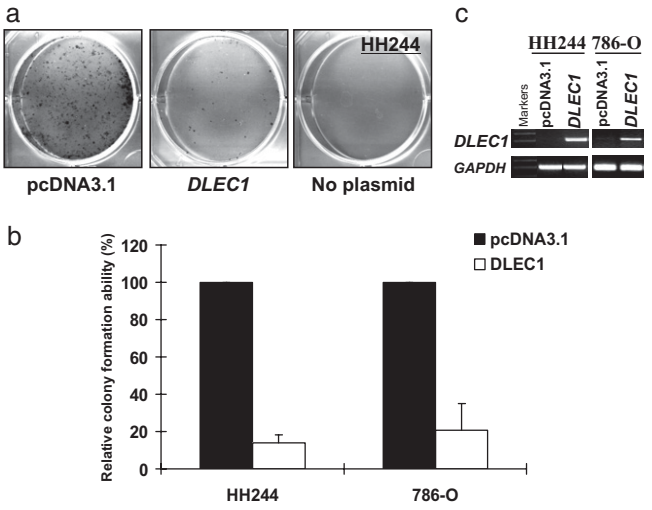

Figure 3. Ectopic *DLEC1* expression suppressed RCC cell clonogenicity. *a*, representative inhibition of colony formation in monolayer culture by *DLEC1* in HH244 cells transfected with pcDNA3.1-DLEC1 or pcDNA3.1 and selected with G418. *b*, colony number quantitative analysis. Number of G418 resistant colonies in vector transfected cells was set to 100%. Values represent mean ± SD of 3 independent experiments. *c*, *DLEC1* ectopic expression was confirmed by 32-cycle RT-PCR.

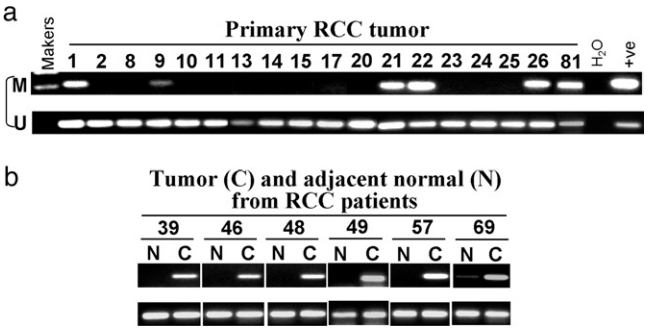

Figure 4. Representative MSP results. *a*, *DLEC1* methylation in RCC. *M*, methylated promoter. *U*, unmethylated promoter. *b*, paired RCC (*C*) and matched normal renal tissue (*N*) samples.

which *DLEC1* methylation was detected by MSP (figs. 4 and 5), densely methylated alleles were detected by BGS. In contrast, few methylated CpG sites were found in RCC-C17 or in normal tissues N49 and N57, which had no methylated band on MSP (figs. 4 and 5).

To evaluate the relationship of *DLEC1* methylation with clinicopathological features in patients with RCC, 81 tumors were divided into 2 subgroups by *DLEC1* methylation status. There was no significant association of *DLEC1* methylation with gender or age (table 1). We then analyzed the relationship of stages pT1, T2 and T3 with *DLEC1* methylation. According to the AJCC 2002 update pT1 stage can be further divided into T1a (tumors 4 cm or less) and T1b (tumors greater than 4 to 7 cm or less). *DLEC1* methylation was significantly more common in more advanced stage tumors (pT1a vs pT1b, pT2 and pT3, $p = 0.01$). We also discovered a positive correlation between *DLEC1* methylation and nuclear grade on statistical analysis ($p = 0.04$).

DISCUSSION

We examined *DLEC1* methylation status in RCC cell lines and tumor tissues. To our knowledge this represents the first study of *DLEC1* epigenetic alteration in RCC and its relationship with clinicopathological features. *DLEC1* methylation and down-regulation were detected in all 9 RCC cell lines and *DLEC1* was methylated in 31% of primary RCCs. The higher methylation rate in cell lines than in primary tumors indicates that some cell lines may have acquired *DLEC1* methylation during the establishment or maintenance process. *DLEC1* methylation also significantly correlated with TNM classification and grade in patients

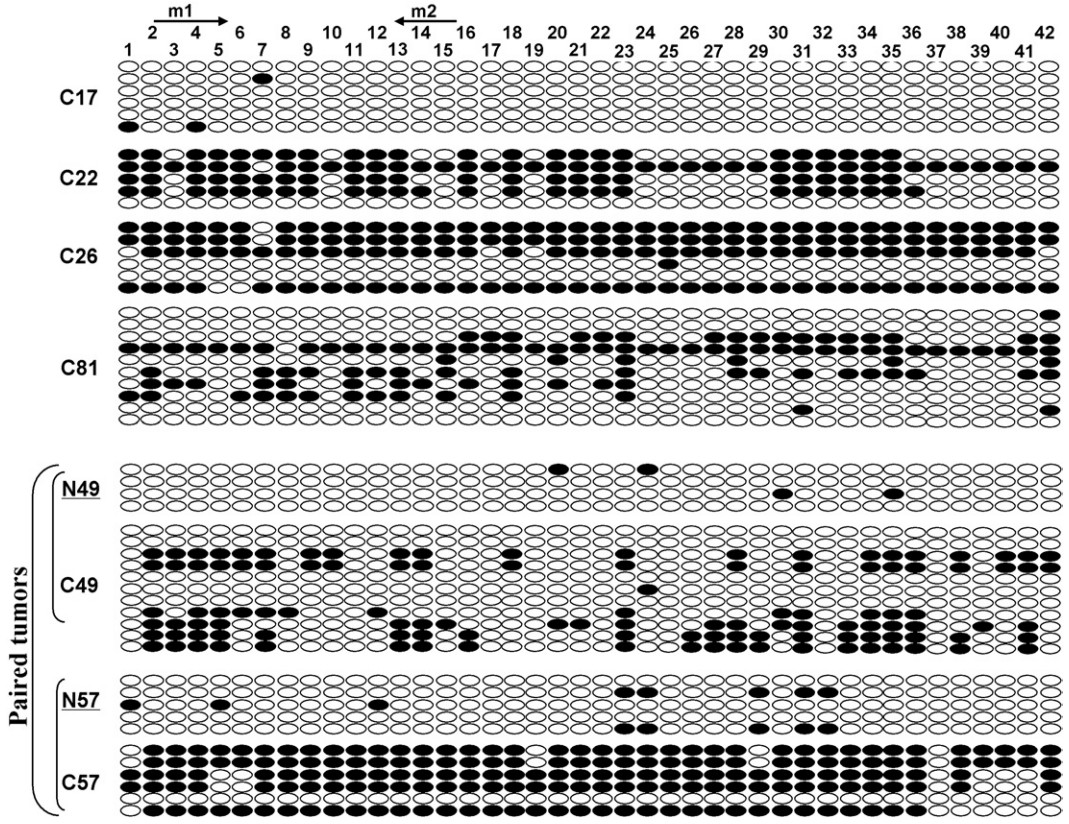

Figure 5. BGS high resolution mapping of *DLEC1* methylation in RCC and paired nontumor samples. Arrows indicate MSP primer sites. Ovals indicate CpG site. Filled ovals indicate methylated. Open ovals indicate unmethylated. Rows indicate individual alleles analyzed.

with RCC but further, larger scale studies are needed to confirm these findings.

The incidence of *DLEC1* methylation in RCC was similar to that in colon (29%) and gastric (34%)[15] cancers but less than in ovarian (54%)[19] and nasopharyngeal (71%) cancers.[20] However, *DLEC1* is still more commonly methylated than other TSGs, including *VHL*, *p16*, *adenomatous polyposis coli*, *glutathione-S-transferase P*, *alternative reading frame*, *death associated protein kinase*, *checkpoint with forkhead and ring finger domains*, *MLH1* and *CDH1*, which are generally methylated in less than 30% of RCCs.[9,10,21]

DNA methylation is a key regulatory factor of gene transcription and genomic stability. DNA methylation alteration is one of the most common epigenetic changes to silence TSGs in cancer,[4] although it is less often observed in RCC. For example, *RASSAF1* is methylated in 27% to 56%,[21] *tissue inhibitor of metalloproteinase-3* is methylated in 58% to 78%,[21] *homeobox B13* is methylated in 73%[9] and *CTNNG/c-catenin* is methylated in 83%[8] of RCCs. Recently we reported *deleted in liver cancer 1* methylation in 35% of RCCs.[11] Epigenetic silencing of these TSGs leads to aberrant cell regulations and contributes to RCC pathogenesis.

DLEC1, located in 3p22.3, contains 37 exons, spans about 59 kb and encodes a 1,755 amino acid protein. *DLEC1* was first identified as a candidate TSG involved in lung and esophageal cancers.[22] Later it was also reported to be commonly downregulated by epigenetic alteration in ovarian cancer[19] and nasopharyngeal carcinoma.[20] Recently our group also reported methylation associated *DLEC1* silencing in hepatocellular, colon and gastric cancers.[14,15] These findings suggest that *DLEC1* is a putative TSG for multiple cancers. In this study *DLEC1* functional significance was further explored by examining the inhibitory effect of *DLEC1* expression in renal tumor cells. Introducing *DLEC1* in silenced cell lines 786-O and HH244 significantly suppressed growth on colony formation assay. A similar tumor suppressive property of *DLEC1* was observed in esophageal, lung, colon, gastric, liver, nasopharyngeal and ovarian tumor cells.[14,15,19,20,22] However, to our knowledge the molecular mechanism underlying this tumor suppressive function of DLEC1 remains unknown. The predicted amino acid sequence of DLEC1 has no significant homology to any known protein or domain while 27 potential CK2 phosphorylation sites are present in DLEC1.[22] CK2 is required at multiple transitions of the cell cycle, including G0/G1, G1/S and G2/M,[14] indicating that DLEC1 may be a CK2 target involved in cell cycle arrest. Our recent study also proved that DLEC1 induced G1 cell cycle arrest in hepatocellular carcinoma cells.[14]

TNM and nuclear grades are commonly used for prognostic prediction in patients with RCC. RCC staging has evolved from the Robson classification to the TNM system, as developed by UICC and AJCC. The most recent revision of the TNM system for RCC, introduced in 2002, further subdivided organ confined tumors, reclassified tumors with venous involvement and clarified the staging of tumors invading perisinus fat.[16] Multiple studies suggest that these revisions have substantially improved prognostic prediction in patients with RCC.[23] Tumor specific TSG promoter methylation can also serve as a tumor biomarker for early diagnosis and prognostication.[24] Our statistical analysis of clinicopathological features in patients with RCC showed a positive correlation between *DLEC1* methylation and pTNM stage or nuclear grade. Similar findings were noted for hepatocellular carcinoma.[14] In conclusion, our results suggest that *DLEC1* inactivation may increase the malignant potential of renal cancer, and detecting *DLEC1* methylation could provide prognostic information on this disease, especially when TSG methylation can be detected in serum and urine samples in patients with RCC, such as *RASSF1A*, *tissue inhibitor of metalloproteinase-3* and *CDH1* methylation.[25]

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