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Cancer Letters 249 (2007) 220–226

CANCER Letters

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Aberrant methylation of the 8p22 tumor suppressor gene *DLC1* in renal cell carcinoma

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Received 24 June 2006; received in revised form 18 August 2006; accepted 25 August 2006

Abstract

Epigenetic mechanisms involving DNA methylation and chromatin remodeling are important in silencing tumor suppressor genes (TSG) in various malignancies, including renal cell carcinoma (RCC). *DLC1* (*deleted in liver cancer 1*)/*ARHGAP7* is a recently identified 8p22 candidate TSG. Frequent methylation of the *DLC1* promoter with resultant gene silencing has been reported in several tumors, but not in RCC yet. We examined *DLC1* promoter methylation in 34 primary RCCs and the corresponding non-malignant tissues, and the correlation of *DLC1* methylation with the clinicopathological characteristics of RCC patients. Although *DLC1* methylation and downregulation were only detected in one of seven RCC cell lines using methylation-specific PCR (MSP) and semi-quantitative reverse-transcription PCR, we found that the *DLC1* promoter was methylated in 35% (12/34) of primary RCC tumors, which was further confirmed by direct sequencing of MSP products and high-resolution bisulfite genomic sequencing. In contrast, only one of the 34 (3%) non-malignant renal tissues had weak methylation. Aberrant *DLC1* methylation appeared to be a relatively early event during renal tumorigenesis since 33% of the RCC tumors with pT1 (TNM staging) showed methylation, which is similar to other late stage tumors. Thus, our results demonstrated that *DLC1* methylation occurs in a subset of RCC tumors and may play a role in renal carcinogenesis.
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Keywords: Methylation; Tumor suppressor gene; *DLC1* gene; Renal; Carcinoma

1. Introduction

Renal cell carcinoma (RCC) is a malignant tumor with poor prognosis and high rate of metastasis to distal organs, accounting for 2% of all adult tumors [1]. Clear cell and papillary carcinoma are the two most common subtypes of RCC, accounting for

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doi:10.1016/j.canlet.2006.08.019

70–80% and 10–15% of all renal carcinomas respectively [2–4]. The molecular mechanisms underlying RCC development remain poorly understood.

Genetic abnormalities have been previously described in RCC. Loss of heterozygosity (LOH) at the 3p25–26 locus involving the *VHL* gene, was the most common genetic event of this cancer [5,6]. Loss of chromosome regions 5q, 6q, 8p12–21.1, 9p21, 10q, 13q 14q24.2-qter and 17p has also been identified in 15–45% of RCC [7–9]. However, inactivation of well-known tumor suppressor genes (TSG) such as *TP53* and *RB1* is uncommon in RCC [10]. In addition to genetic changes, epigenetic inactivation of TSGs has also been well described to play important roles in carcinogenesis [11]. One mechanism of epigenetic inactivation is the aberrant methylation of CpG islands (CGI) of TSG promoters, which leads to the binding of transcription repressors, compressed chromatin, and transcription silencing [11]. A growing list of aberrantly methylated TSGs has been reported in RCC, including *VHL*, *p16INK4A*, *APC*, *GSTP*, *DAPK*, *CDH1*, *RASSF1A*, *DAL-1/4.1B*, *γ-catenin, SPINT2* and *HOXB13* [12–18]. However, most of these TSGs have a relatively low frequency of methylation in RCC. Thus, further studies of more TSGs are needed to identify putative epigenetic biomarkers for this tumor.

The *deleted in liver cancer 1* (*DLC1*) gene at 8p22 is a candidate TSG, homologous to rat *p122 RhoGAP* [19]. DLC1 can activate GTP-bound and inactivate GDP-bound forms to determine the activities of Rho proteins. The deregulation of Rho GTPase proteins is critical to tumor pathogenesis [20]. Multi reports have shown that the transcriptional silencing of *DLC1* by aberrant promoter methylation is commonly involved in the pathogenesis of prostate, breast, lung, colon, gastric and liver carcinoma [21–26]. Recently, our group also demonstrated the frequent methylation of *DLC1* in nasopharyngeal, esophageal, cervical and breast carcinomas [27]. As the *DLC1* locus is also a region affected in RCC [9], we investigated whether aberrant methylation of *DLC1* also contributes to renal cell carcinogenesis.

2. Materials and methods

2.1. Cell lines and tumor samples

Seven RCC cell lines (A498, 786-O, CaKi, HH050, HH244, RCC52 and RCC98) (from ATCC or S-K Liao) and the normal embryonic kidney cell line HEK293 (from ATCC), which serves as a "normal" control for RCC, were routinely maintained in RPMI or DMEM medium with 10% fetal bovine serum.

Thirty-four primary renal cell carcinomas and their corresponding non-malignant renal tissues were obtained from the Urology Department of Peking University First Hospital, Beijing, China (from January to September, 2005), with patients' consent according to the university policy. The male to female ratio of these patients was 1.61:1 (21:13), and the age range was 21–73 years (mean age, 49.5 years). 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 −80 °C. Pathological diagnosis was performed and confirmed at the Pathology Department, Institute of Urology, Peking University First Hospital. Tumors were histopathologically classified according to the criteria of TNM (tumor node metastasis) stages [3].

2.2. DNA and RNA extraction

For DNA extraction, 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 EDTA, 10 mM NaCl, 2% *N*-lauryl sarcosyl and 200 μg/ml proteinase K for 20 h at 55 °C, followed by phenol–chloroform extraction and ethanol precipitation. Total RNA and DNA were extracted from cell line pellets using TRI Reagent (Molecular Research Centre, Inc., Cincinnati, OH) according to the manufacturer's protocol [28,29]. Genomic DNA samples from one nasopharyngeal carcinoma cell line CNE1 and 5-aza-2′-deoxycytidine (Aza)-treated CNE1 were used as controls for methylation analysis [27].

2.3. Bisulfite treatment and promoter methylation analysis

Bisulfite modification of DNA was carried out as described previously [30]. Methylation-specific PCR for the *DLC1* promoter was carried out in an MJ DNA Engine Dyad® Thermal Cycler (MJ research, Waltham, MA), in a 12.5 μl PCR mixture containing 0.5 μl of bisulfite-treated genomic DNA, dNTPs (each at 200 μM), primers (0.6 μM each), 2 mM $MgCl_2$, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA) in 1× PCR II Buffer [31]. The bisulfite-treated DNA was amplified with the methylation-specific primer set [27], DLC1bm11: 5′-AACCGAAAAACAACCCG TCG-3′, DLC1bm22: 5′-GAGCGAATTGTTTTTCG CGC-3′, or the unmethylation-specific primer set, DLC1bu11: 5′-AAAACCAAAAAACAACCCATCA-3′, DLC1 bu22: 5′-TTGGAGTGAATTGTTTTTTGTGT-3′. The predicted size of the unmethylated amplicon is 144 bp, and the methylated amplicon is 139 bp. The PCR conditions were as follows: initial denaturation

and hot start for 10 min at 95 °C, then 40 cycles consisting of 30 s at 94 °C, 30 s at 60 °C (for methylated reactions) or 58 °C (for unmethylated reactions), and 30 s at 72 °C, and a final extension of 5 min at 72 °C. MSP primers were tested previously for not amplifying any unbisulfited DNA, and the MSP products of several primary tumors have been confirmed by direct sequencing with BigDye v3.1 (Applied Biosystems), indicating that our MSP system is specific [27]. Bisulfite genomic sequencing (BGS) were performed as previously described [30,31], using primers (for the bottom strand) DLC1BGSb1: 5′-CCAAATAAATACCTTA TAACCTTTA and DLC1BGSb2: 5′-GAGGTG YGGTTATGTTTTGGT (Y = C or T). Amplified BGS products were TA-cloned and 5–12 colonies were randomly chosen and sequenced.

2.4. Statistical analysis

Statistical analysis was performed using the Fisher's exact test, Student's t test, and the χ^2 test; $P < 0.05$ was considered as statistically significant.

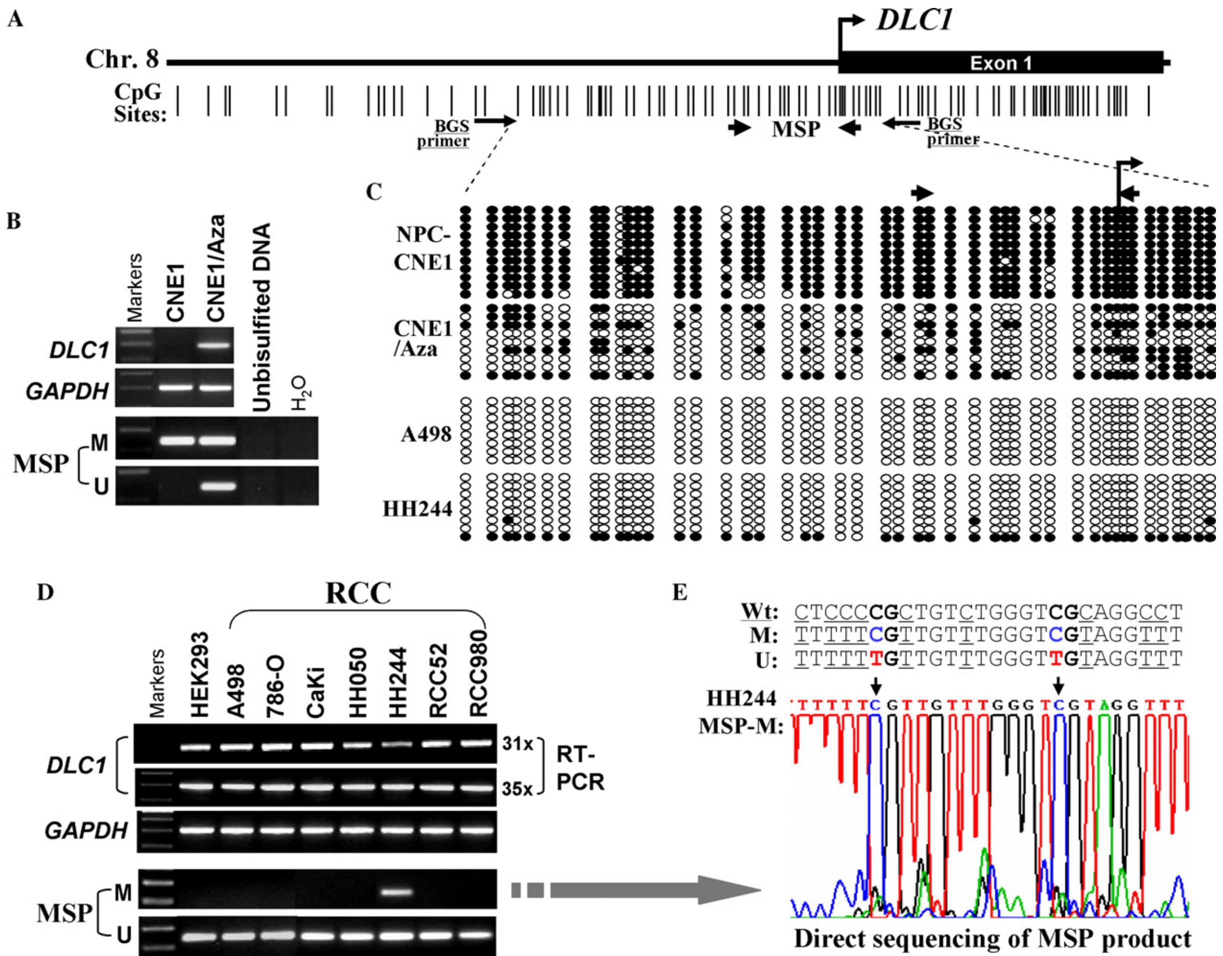

Fig. 1. Analysis of *DLC1* methylation by methylation-specific PCR (MSP). (A) Schematic diagram of the *DLC1* promoter CpG island (CGI). The core promoter, exon 1 (black rectangle) and CpG sites in the CGI (short vertical lines) are shown. The transcription start site (NM_006094) is indicated by a curved arrow. The MSP and BGS regions analyzed are indicated. (B) Bisulfite-modified genomic DNA from CNE1, CNE1 treated with Aza (10 μM for 3 days) or un-bisulfited DNA, was used in MSP reactions with unmethylation (U) or methylation-specific (M) MSP primer sets. Expression levels of *DLC1* as detected by RT-PCR in these cell lines were also shown, *GAPDH* was used as an RT-PCR control (top two panels). (C) High-resolution mapping of the methylation status of every CpG site in the *DLC1* promoter by BGS. Each row of circles (CpG sites) represents an individual allele of the *DLC1* promoter analyzed. The transcription start site is indicated by a curved arrow, MSP primer sites by arrows. Black filled circle, methylated; empty circle, unmethylated. (D) Expression and methylation of *DLC1* in RCC cell lines by RT-PCR and MSP. M, methylated; U, unmethylated. (E) The MSP product (M) was directly sequenced and confirmed. Methylated cytosines (C) would not be converted to uracil (T) and remained as C. On the top panel, the wild-type sequence (WT) without bisulfite conversion is shown.

3. Results

To analyze the methylation status of the *DLC1* promoter in RCC, we designed a set of MSP primers that amplify a segment of its promoter, targeting either methylated or unmethylated promoter alleles (Fig. 1) [27]. We tested the MSP primers in genomic DNA from one nasopharyngeal carcinoma cell line CNE1 and Aza-treated CNE1. Only methylated product was obtained in CNE1 while both methylated and unmethylated products obtained in Aza-treated CNE1 (Fig. 1B), consistent with our previous report [27]. Furthermore, no amplification was detected in genomic DNA without bisulfite modification, indicating that our MSP system is specific. The methylation status of these cell lines was further confirmed by high-resolution BGS analysis (Fig. 1C). Unmethylated promoter alleles were obtained after Aza-treatment, along with the activation of *DLC1* expression (Fig. 1B), indicating that methylation is the direct factor silencing the *DLC1* promoter [27].

We then used this validated system to examine the methylation status of *DLC1* in RCC cell lines. MSP results showed that only one (HH244) out of 7 RCC cell lines had weak methylation, while unmethylated promoter was detected in all 7 RCC cell lines as well as the normal embryonic kidney cell line HEK293 (Fig. 1D). Direct sequencing of the methylated PCR product confirmed the MSP result of HH244 (Fig. 1E). This result correlated with the expression levels of *DLC1* in these cell lines, with downregulation of *DLC1* detected in HH244 (detectable only with less cycles of RT-PCR) but not in other cell lines (Fig. 1D). Furthermore, the *DLC1* methylation status of two RCC cell lines HH244 (partially methylated) and A498 (unmethylated) was further confirmed by BGS. The BGS results fully confirmed our MSP data, with no methylated CpG site detected in A498 but 13% of CpG sites methylated in HH244 (Fig. 1C).

We then analyzed 34 primary RCC specimens and their corresponding non-malignant renal tissues. The clinicopathological features of these patients and the MSP result are summarized in Table 1 and Fig. 2. *DLC1* methylation was detected in 12 of 34 (35%) RCC tumors. In primary tumors, the background normal stromal cells would contribute to the unmethylated band in all cases, thus unmethylated alleles were detected in all tumor and non-malignant samples. In the non-malignant tissues, only 1 of 34 (3%) samples showed weak methylation. Representative MSP results of RCC and their corresponding non-malignant renal tissues are shown in Fig. 2A. MSP products were further confirmed by direct sequencing, as shown in Fig. 2B. High-resolution BGS analysis of 3 tumors and 1 non-malignant tissue also confirmed their methylation status in detail (Fig. 2C).

The relationship of *DLC1* methylation with the clinicopathological features of these patients was also analyzed. As shown in Table 1, there was no significant correlation of *DLC1* methylation with gender, age, tumor site, TNM stage or nuclear grade, by statistical analyses. *DLC1* methylation was observed in 7 of 21 (33%) tumors with stage pT1, and the percentage of methylation did not increase significantly in tumors with more advanced stages, suggesting that *DLC1* methylation occurs in a relatively early stage during the multi-step renal carcinogenesis.

Table 1
Clinical features of RCC patients and their methylation status for *DLC1*

Clinicopathological features	*DLC1* promoter		
	Methylated (%)	Unmethylated (%)	*p*-value
Cases	12 (35)	22 (65)	
Age (mean, yr)	47.4	53.3	NS[*,a]
Gender			
Male	8 (38)	13 (62)	NS[b]
Female	4 (31)	9 (69)	
Site			
Right	5 (33)	10 (67)	NS[b]
Left	7 (37)	12 (63)	
TNM classification			
pT1	7 (33)	14 (67)	NS[c]
pT2	3 (38)	5 (62)	
pT3	2 (40)	3 (60)	
pT4	0	0	
Nuclear grade			
G1	3 (33)	6 (67)	NS[c]
G2	7 (39)	11 (61)	
G3	2 (29)	5 (71)	

[a] Student's *t* test.
[b] Fisher's exact test.
[c] χ^2 test.
[*] NS, not significant.

4. Discussion

In this study, we examined the methylation status of *DLC1* in RCC using a highly specific and sensitive MSP method. To our best knowledge, this report represents the first study to evaluate the epigenetic alterations of *DLC1* in RCC samples. Although methylation and downregulation of *DLC1* were only detected in one of seven RCC cell lines examined, we found that *DLC1* methylation occurs at a subset (35%) of primary RCC tumors, and the aberrant methylation appeared to be a relatively early event in renal carcinogenesis.

Tumorigenesis is a multistep process due to the accumulation and interplay of genetic and epigenetic alterations. DNA methylation is a key regulator

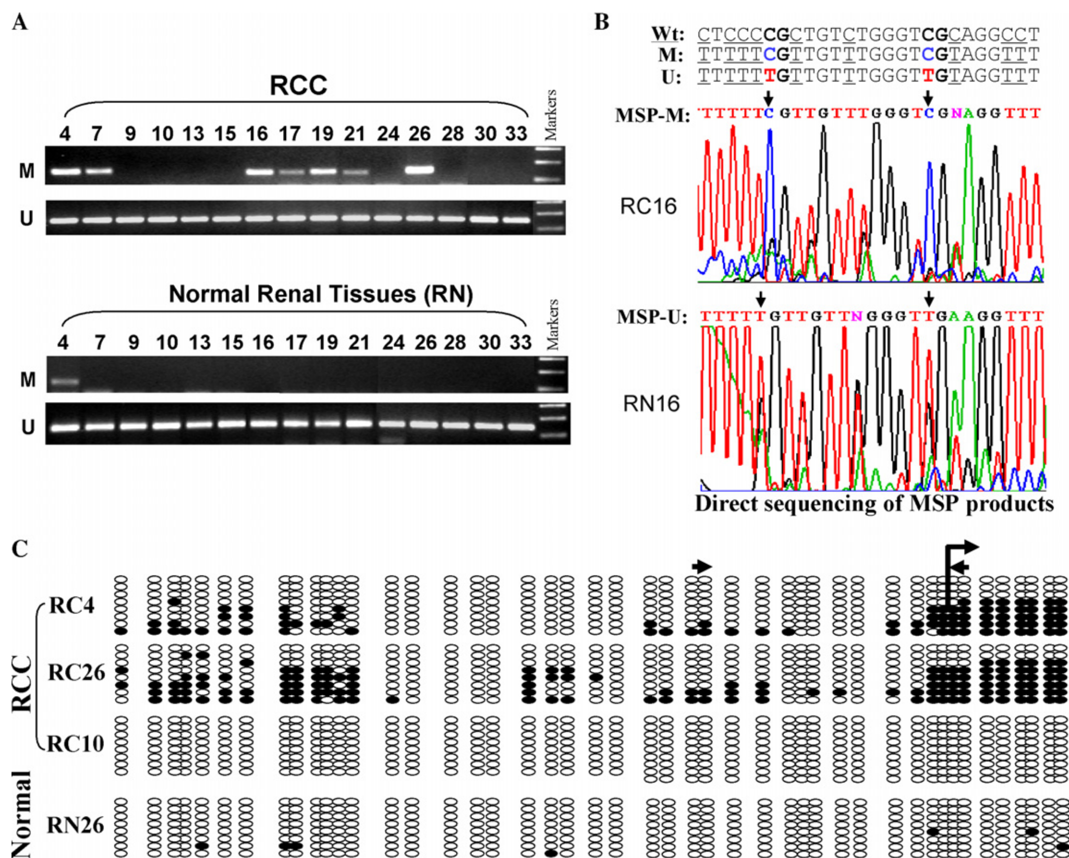

Fig. 2. Representative MSP and BGS results of *DLC1* methylation in RCC tumors. (A) The MSP results of 15 RCC samples and their matched normal renal tissues. U, MSP for unmethylated promoter; M, MSP for methylated promoter. (B) The MSP products were further directly sequenced and confirmed. (C) High-resolution mapping of the methylation status of every CpG site in the *DLC1* promoter by BGS in tumor and non-tumor samples. The *DLC1* transcription start site is indicated by a curved arrow.

of gene transcription and genomic stability. Alteration of DNA methylation is one of the most consistent epigenetic changes to silence TSGs in human cancers [11]. Aberrant methylation also results in increased gene mutagenicity, due to the deamination of 5-methylcytosine to thymine [11]. In RCC, methylation of promoter CpG islands and decreased expression of several TSGs have been reported, with most of them infrequently methylated [12–18]. *RASSFA1* is methylated in 27-56% [17,32,33], *TIMP3* methylated in 58–78% [32,34], *HOXB13* methylated in 73% [12], and *CTNNG/JUP/γ-Catenin* methylated in 83% [15], of primary RCC respectively. Unlike other carcinomas, the frequencies of aberrant methylation of most classical TSGs, such as *VHL, p16*, *APC*, *GSTP*, *ARF*, *DAPK*, *CHFR*, *MLH1* and *CDH1*, are less than 30% in RCC [12,16,32,35], suggesting that these genes are probably not the major epigenetic targets for methylation silencing in RCC. Our results showed *DLC1* methylation in 35% (12/34) of RCC, also less frequent than that of other carcinomas [21–27], but similar to that in breast cancer [27]. Thus, other TSGs and cancer genes important in renal tumorigenesis likely remain to be identified for their epigenetic alterations in RCC.

DLC1 is a recently identified TSG with a RhoGAP functional domain [19,36], which specifically catalyzes the conversion of the active GTP-bound RhoA protein, a member of the Rho small GTP-binding subfamily proteins, into the inactive GDP-bound protein [20]. Since active RhoA protein is involved in Ras-mediated tumorigenic transformation, *DLC1* thus function, through its RhoGAP domain, to inhibit tumorigenesis [20]. Recent evidence has shown that *DLC1* meets several criteria of being a classic TSG: located at 8p21.3–22 which is frequently deleted in tumors, including breast, colon, liver, lung and prostate cancers [19]; and frequently, downregulated in multiple tumors due to

aberrant methylation. Additional evidence supporting *DLC1* as a TSG came from the observations of its ability to inhibit various tumor cell growth and colony formation [22,24,27,37]. Furthermore, transfection of *DLC1* into breast and lung cancer cells without expression led to the prevention of tumor formation in athymic nude mice [24,26], indicating that *DLC1* is a *bona fide* TSG for multiple tumors.

In summary, our study provides the first documentation that *DLC1* is methylated in a subset of RCC tumors. *DLC1* methylation is tumor-specific and occurs at the early stage of RCC pathogenesis. Our results suggested that epigenetic silencing of *DLC1* play a role in renal carcinogenesis, and may be used as a biomarker for RCC diagnosis.

Acknowldgments

Supported by a National Natural Science Foundation of China (NSFC) Grant (#30600612) and the Chinese University of Hong Kong.

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