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Original Article

Identification of hypomethylation profile of Ki-67 promoter in vitro and in vivo

Liantao Li[1,2*], Guowei Qian[1,2,3*], Hui Tian[2], Jiehui Di[2], Feifei Chen[2], Junnian Zheng[1,2]

[1]Jiangsu Key Laboratory of Biological Cancer Therapy, Xuzhou Medical College, Xuzhou 221002, Jiangsu, PR China; [2]Center of Clinical Oncology, Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, Jiangsu, PR China; [3]Department of Breast Surgery, Fudan University Shanghai Cancer Center, Shanghai 200000, China. [*]Equal contributors.

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Abstract: Ki-67 is widely used as a proliferation marker in cancer diagnosis, but little is known about its gene regulation. CpG methylation is a common epigenetic modification distributed at cis-regulatory regions and makes variable impacts on transcription. To determine whether DNA methylation is involved in Ki-67 gene regulation, we performed bisulfite sequencing and methylation specific PCR (MSP) to generate the methylation profile of Ki-67 promoter in vitro and in vivo, respectively. In normal and cancer cell lines with high expression of Ki-67 gene, we identified that Ki-67 promoter was hypomethylated. Besides, in clear cell renal cell carcinoma (ccRCC) specimens and matched normal renal tissues with different expression level of Ki-67 gene, an unmethylated profile of Ki-67 promoter was also observed. Our data indicated that methylation status of Ki-67 promoter played little role in its gene regulation.

Keywords: Ki-67, DNA methylation, CpG Island, clear cell renal cell carcinoma

Introduction

Ki-67 protein (pKi-67) was originally described as a nuclear protein which is necessary for cell proliferation [1]. It was only present in active phases of cell cycle (G1, S, G2 and mitosis), but not in quiescent cells (G0). Although an extensive knowledge of pKi-67 structure has been achieved, it is difficult to determine its function owing to the lack of homologous proteins. The most general function speculated for pKi-67 is regulating cell cycle because it shares the similar structure (FHA domain) with other cell cycle regulators [2]. The requirement of pKi-67 for cell cycle progression was confirmed by the outcome induced by expression of pKi-67 targeted antisense oligonucleotides, microinjection of anti-pKi-67 antibodies, or overexpression of recombinant pKi-67 tandem repeats [3-5].

Uncontrolled cell proliferation is a hallmark of malignancy [6]. Exclusive expression in proliferating cells makes pKi-67 an excellent marker to assess proliferation status of tumor cells. It has been widely used as a prognostic and predictive marker for many cancers, especially for prostate and breast carcinomas [7]. In addition, multiple studies have reported that Ki-67 could serve as a potential target for cancer therapy. For example, Kausch et al. demonstrated that antisense-mediated inhibition of Ki-67 expression led to significant inhibition of cell proliferation and tumor growth in vitro and in vivo [8]. One previous study in our laboratory has proved that renal cancer cell growth can be inhibited in vitro and in vivo by oncolytic adenovirus armed short hairpin RNA targeting Ki-67 encoding mRNA [9]. Together, these findings suggested that Ki-67 might play a crucial role in tumorigenesis.

Past few years have witnessed huge progress in the study of cancer epigenetics. One of the best-described epigenetic events is DNA methylation, which plays a pivotal role in carcinogenesis through epigenetic regulation of cancer-related genes. In general, cancer cells are characterized by global genomic hypomethylation accompanied by regional hypermethylation of CpG islands at specific gene promoters, especially at promoters of tumor suppressor genes [10]. Altered DNA methylation pattern has been found as a hallmarker of cancer cells, this made

it a power tool in tumor diagnosis and prognosis evaluation [11]. The presence of a large CpG island with a dense CG-rich content at Ki-67 promoter region suggests that Ki-67, as a cancer-related gene, may be regulated by DNA methylation.

Our present report focuses on assessing the methylation status of Ki-67 promoter in normal, cancer cell lines, and primary ccRCC tissues, in order to investigate a possible role for DNA methylation in the regulation of Ki-67 gene.

Materials and methods

Cell culture and tumor samples

Human umbilical vein endothelial cell line (HUVEC), human normal liver cell line (HL-7702), human lung adenocarcinoma cell line (A549), human cervical adenocarcinoma cell line (Hela), and human gastric adenocarcinoma cell line (SGC7901) were obtained from Shanghai Cell Collection (China), human bladder transitional cell carcinoma cell line (EJ) and human renal cell carcinoma cell line (Kert-3) were purchased from Nanjing KeyGen Biotech (China). All cells were cultured in RPMI Medium 1640 (Invitrogen) supplemented by 10% fetal bovine serum (FBS, Invitrogen) except for Hela cells, which were grown in Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% FBS. For serum starvation, cells were maintained in medium with 0.2% FBS for 72 h and were then harvested for experimental use. All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. 24 ccRCC tissues and matched normal renal tissues were collected from Urology Department of Affiliated Hospital of Xuzhou Medical College with written informed consent and approved by the Ethics Committee of Xuzhou Medical College; none of the patients had a prior history of ccRCC or received any adjuvant therapy, specimens were snap-frozen in liquid nitrogen or immediate-fixed in paraformaldehyde. The clinic-pathological features of the patients are listed in Table S1.

RNA extraction and RT-PCR

Total RNA was isolated from cell lines using RNeasy Protect Cell Mini Kit (Qiagen), and about 500 ng RNA was reverse transcribed into cDNA using PrimeScript RT reagent Kit (TaKaRa). PCR was carried out with three primer sets specific for β-actin, Ki-67, or hTERT respectively in a final volume of 25 μL, containing 1 μL of cDNA, 1 μL of each primer, 12.5 μL of 2×GoTaq Green Master Mix (Promega), and 9.5 μL of nuclease-free water. All the primers were listed in Table S2. Amplication conditions were as follows: the initial denaturation (95°C, 2 min) was followed by 30-35 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, and a final extension step at 72°C for 5 min. PCR products were separated on 2% agarose gel.

Real-time PCR

Ki-67 RT-PCR products were subcloned into pGEM-T Easy vectors (Promega), and the constructs were then verified by sequencing. After plasmid midi preparation, the circular constructs were linearized by EcoRI (New England Biolabs) outside of the intended PCR region and were then purified using Wizard SV Gel Clean-Up System (Promega). To generate the standard curve, six serial dilutions of linearized plasmid DNA ranging from 10^3 to 10^8 copies/μL were prepared as standards.

The absolute quantification of transcript levels for Ki-67 gene was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystem). Primers were the same with RT-PCR. The sequences of TaqMan probes were: 5'-(FAM) TCAAGAAAGACAAAAAGCCAGCCTGCAG(DABCYL)-3'. PCR reaction mixture was prepared as follows: 1 μL of cDNA, 0.4 μL of each primer (10 μM), 0.4 μL of 50×ROX Reference Dye (TaKaRa), 0.8 μL of Taqman probe, 10 μL of 2×Premix Ex TaqTM (TaKaRa), and 7 μL of nuclease-free water. The thermal cycling profile consisted of initial denaturation at 95°C for 30 s and 40 cycles at 95°C for 5 s, 60°C for 15 s. PCR products were inspected on a 2% agarose gel. Each sample was processed in triplicate.

Immunohistochemistry

All the specimens were formalin-fixed and paraffin-embedded. Several 4 mm thick sections were cut for each sample. The detailed methods for immuostaining and scoring for pKi-67 were previously described [9].

CpG Island prediction

CpG island was identified by screening the 5'-flanking sequence (-2000 to +1000, relative to the transcription start site, TSS) of Ki-67 gene using online program CpG Island Searcher

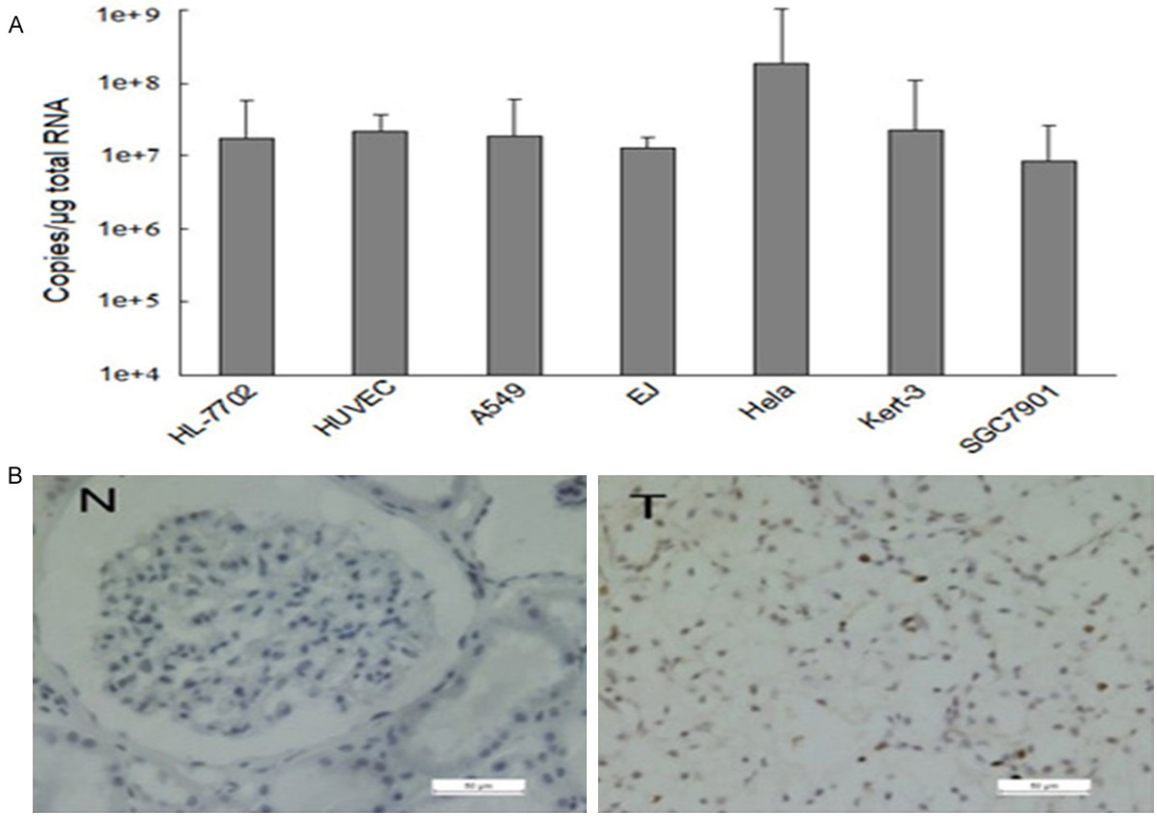

Figure 1. A. Quantitation of Ki-67 mRNA copies in 1 μg of total RNA extracted from normal and cancer cell lines. Bars indicate SD. B. Representative immunostaining of pKi-67 in ccRCC tissues (T) and matched adjacent normal renal tissues (N). Bar, 50 μm; magnification, 400×.

with the following setting parameters (length ≥ 200 bp, GC% ≥ 50%, CpG Obs/Exp ratio ≥ 60%).

Genomic DNA extraction and bisulfite sequencing

Genomic DNA was extracted by QIAamp DNA mini kit (Qiagen), and about 2 μg DNA was subjected to bisulfite conversion with the EpiTect Bisulfite Kit (Qiagen). Bisulfite-modified DNA was used as templet in the subsequent PCR. Two primer sets (P1/P2 and P3/P4) were designed to amplify Ki-67 promoter region (-335 to -17 and -39 to +329, relative to TSS) respectively. The primers were summarized in Table S2. PCR reaction mixture was described as follows: 4 μL of DNA, 1 μL of each primer (10 μM), 25 μL 2×GoTaq Green Master Mix (Promega), and 19 μL of nuclease-free water. PCR was performed in the following conditions: 95°C for 5 min, followed by 35-40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and 72°C for 5 min. The resulted amplicons were subcloned into the pGEM-T Easy vectors (Promega). Eight to ten individual clones of each amplicon were isolated and subjected to sequencing. Alignment of experimental sequences to original Ki-67 genomic sequence was performed using the software CpG viewer to reveal methylation status of Ki-67 promoter.

Methylation specific PCR

Genomic DNA of ccRCC tissues and matched normal renal tissues was extracted and bisulfite-modified as mentioned above. MSP was performed using the EpiTect MSP Kit (Qiagen). 4 μL of bisulfite-modified DNA was used as the template. Two different primers sets (M1/M2 and U1/U2) specific for the methylated (M) and unmethylated (U) Ki-67 promoter sequences were designed (Table S2). PCR reaction conditions were as follows: hot start for 10 min at 95°C, followed by 39 cycles consisting of 15 s at 94°C, 30 s at 52°C and 30 s at 72°C, and a final extension of 10 min at 72°C. Nuclease-free water, CpGenome universal unmethylated and methylated DNA (Millipore) was used as

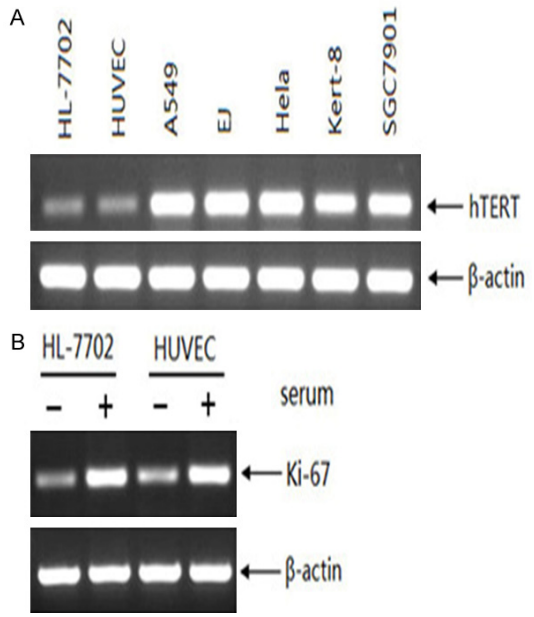

Figure 2. A. Expression analysis of hTERT in normal and cancer cell lines. B. Serum starvation reduced Ki-67 mRNA expression level. HL-7702 and HUVEC cells were cultured in RMPI 1640 containing 10% FBS (+, normal) or 0.1% FBS (-, serum starvation), then total RNA were extracted and subjected to RT-PCR analysis.

blank, negative and positive control, respectively. MSP products were analyzed on a 2% agarose gel.

Results

Analysis of Ki-67 expression in normal, cancer cell lines and primary ccRCC tissues

Ki-67 expression in normal cell lines (HL-7702, HUVEC) and cancer cell lines (A549, EJ, Hela, Kert-3, SGC7901) was quantified by real-time PCR, as shown in **Figure 1A**, all the cell lines used here had high expression level of Ki-67 gene. Since Ki-67 is a proliferation marker, this result suggested the two normal cell lines entered a state of proliferation. It was reported that human normal cells could be transformed into immortalized cells during the culture due to the activation of telomerase, whose activity could be restored by ectopic expression of hTERT, the catalytic subunit of telomerase [12, 13]. Therefore hTERT expression patterns were determined in the two normal cell lines using RT-PCR, the results confirmed its expression in the two cell lines (**Figure 2A**). Serum has been reported to contain the competence factors required to rescue the cells from the quiescent state stimulating them to grow and resume proliferation [14]. So the effect of serum starvation on Ki-67 mRNA expression in the two normal cell lines was evaluated by RT-PCR, results showed that serum starvation decreased Ki-67 expression levels in these two normal cell lines but had no effect on β-actin (**Figure 2B**). Ki-67 expression in primary tumors was evaluated by immunohistochemistry, as shown in **Figure 1B**, ccRCC tissues had strong nuclear staining of pKi-67, while faint staining was observed in adjacent normal renal tissues.

Methylation status of Ki-67 promoter in normal and cancer cell lines

To investigate the role of DNA methylation in regulating Ki-67 gene, the methylation profile of its promoter was analyzed first. A CpG island (-742 to +978, relative to TSS) was identified by the online program CpG island searcher (**Figure 3A**). The detailed information about this CpG island was as follows: length =1720 bp, GC%=61.6%, CpG Obs/Exp=0.897. Then, we selected a region (-335 to +329) that covers the core promoter sequences of Ki-67 gene (-223 to +12, relative to TSS) for methylation analysis [15]. Methylation status of a total of 82 CpG sites located in this region was characterized by bisulfite-sequencing, the results displayed that most of these CpG sites were unmethylated in both normal and cancer cell lines that express high level of Ki-67 gene (**Figure 3B**). The difference in the number of methylated CpG sites among individual clones may result from internal heterogeneity of these cell lines and selection bias in picking up individual clones.

Methylation status of Ki-67 promoter in ccRCC tissues and matched adjacent normal renal tissues

Previous studies had reported that pKi-67 was a biomarker in ccRCC, higher Ki-67 expression is associated with higher nuclear grade and worse prognosis in ccRCC [16]. Besides, we demonstrated that the methylated oligonucleotide targeting Ki-67 promoter remarkably inhibited Ki-67 expression and proliferation of the human 786-0 renal carcinoma cells and induced apoptosis of 786-0 cells [17]. Therefore,

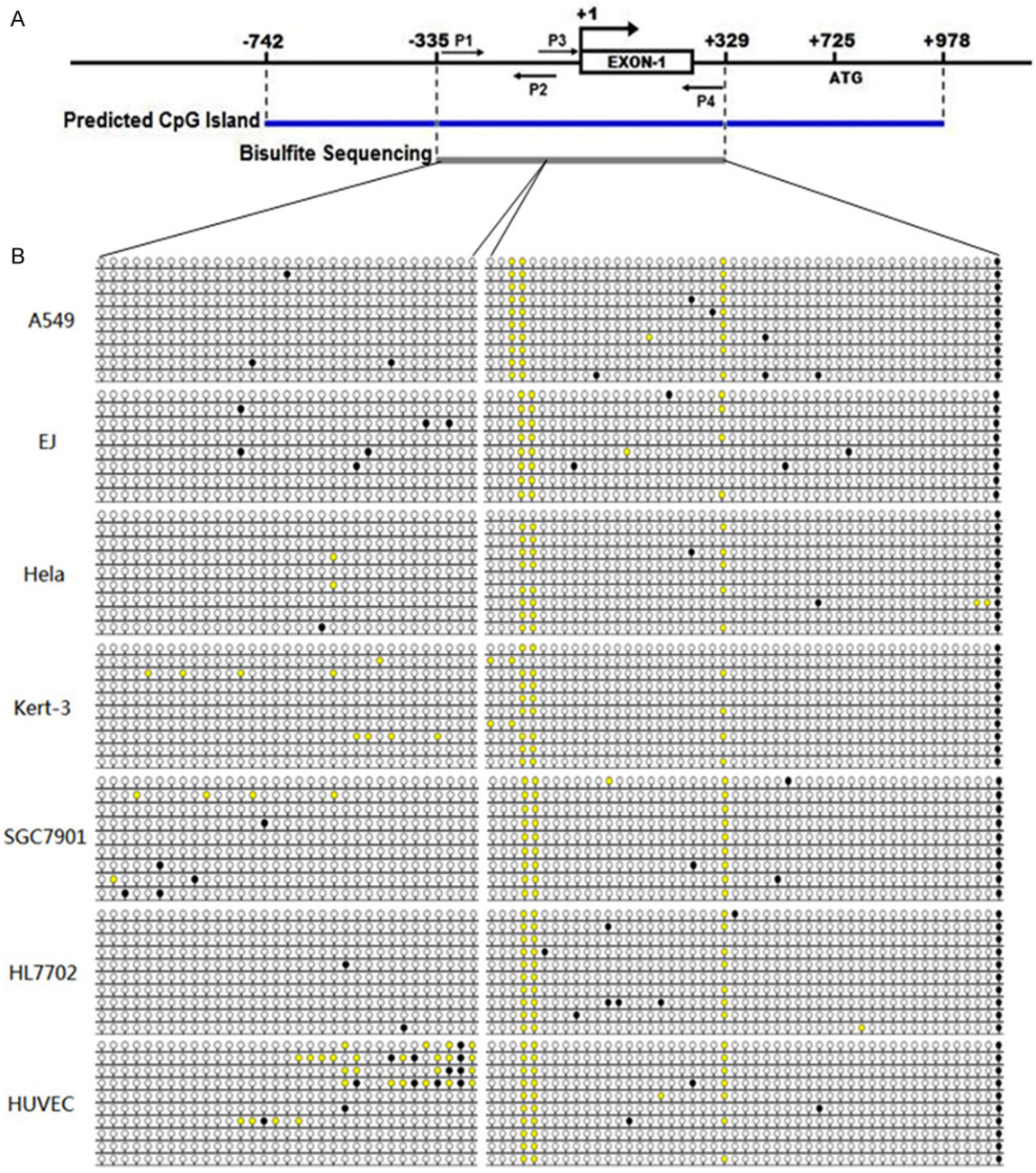

Figure 3. A. The schematic drawing of CpG island (-742 to +978) at Ki-67 promoter region. B. Methylation map of 82 CpGs located in the promoter region from -335 to +329. Each "lollipop" represents a single CpG site. White, unmethylated CpGs; black, methylated CpGs; yellow indicated the presence of a SNP at the position, or a sequencing or PCR artifact.

we attempt to explore whether Ki-67 methylation patterns can also serve as a biomarker in ccRCC, 24 primary ccRCC specimens and their corresponding normal renal tissues were collected for Ki-67 methylation analysis using MSP. To our surprise, Ki-67 unmethylation was detected in 100% of 24 ccRCC tissues and matched normal tissues, although they have remarkable difference in Ki-67 expression level (**Figure 4**).

Discussion

For more than 20 years, pKi-67 has been widely used as a proliferation maker in histology, it is not well understood but familiar to research-

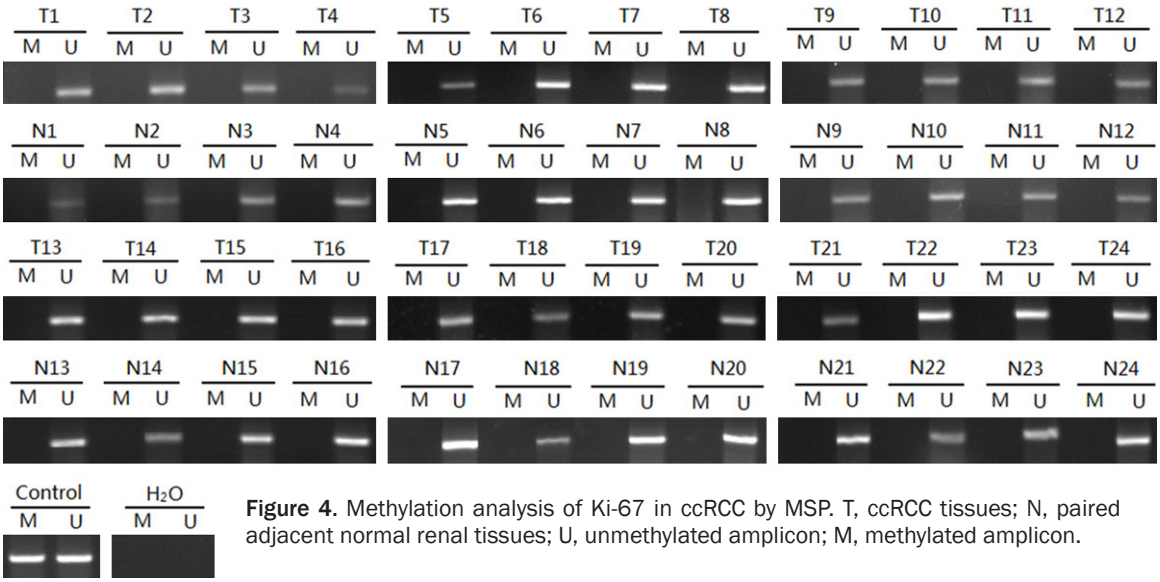

Figure 4. Methylation analysis of Ki-67 in ccRCC by MSP. T, ccRCC tissues; N, paired adjacent normal renal tissues; U, unmethylated amplicon; M, methylated amplicon.

ers. Previously we have made a genetic analysis of Ki-67 promoter. Here we explored the epigenetic regulation of Ki-67 gene in normal and cancer cells.

Concerning Ki-67 gene expression, our results showed that Ki-67 was highly expressed in normal cell lines (HL-7702, HUVEC). In fact, Ki-67 could also be detected in other normal cell lines such as HEK-293 and HFL-I in our laboratory (data not shown). Our data suggested that immortalization and serum stimulation were two possible reasons responsible for the high expression of Ki-67 in normal cell lines. Rahmanzadeh et al. addressed that pKi-67 could be detected not only in proliferating cells but also in quiescent cells, which would not argue against the use of pKi-67 as a proliferation marker [18]. According to this report, it's no wonder that Ki-67 could be detected in both normal and cancer cells. In addition, Ki-67, as a nuclear protein, has been reported to have a DNA binding activity [19] and play a crucial role in ribosomal RNA synthesis [18]. Therefore, there is the possibility that Ki-67 would be a kind of transcription factor whose transcriptional target genes are involved in regulating the growth of both normal and cancer cells.

We investigated the DNA methylation status of a portion of the CpG Island at Ki-67 promoter in vitro and in vivo. We didn't find differences in Ki-67 methylation patterns between normal and cancer cells. This result is similar to methylation patterns of survivin promoter, which is unmethylated in primary acute myeloid leukemia samples and normal peripheral blood mononuclear cells [20]. There is the possibility that the methylation status of Ki-67 promoter play little role in tumorigenesis and that tissue-specific expression of Ki-67 in vivo may be mediated by methylation-independent mechanisms. In order to obtain more detailed information of Ki-67 methylation profile, genome-wide methylation screening is necessary to undertake, and a large number of samples should be pooled.

It is proposed that DNA hypomethylation patterns result from a historical event of transient DNA demethylation and transcriptional factors contribute to determining which regions escape remethylation and remain therefore unmethylated [21]. Sp1 is a ubiquitous transcription factor which plays positive or negative role in regulation of gene expression. On one hand, Sp1 can protect CpG islands from methylation presumably by binding to CpG-rich sequences and denying access to DNMTs [22], on the other hand, Sp1 can recruit histone acetyltransferase P300 leading to acetylation of histone, which would also protect DNA from methylation [22, 23]. Recently, one work of Gebhard and colleagues has revealed that CpG Islands that remain unmethylated in normal and malignant cells contain specific sequence motifs that are identical to consensus sequences for general transcription factors such as Sp1 [24]. Previously, we identified three Sp1-binding sites at Ki-67 gene promoter, and confirmed that Sp1 played a positive role in the regulation of Ki-67 gene expression [25]. Therefore, we can make

a suggestion that hypomethylation of Ki-67 gene promoter is partially due to Sp1-binding. Besides, Weber et al. have reported that strong CpG island promoters are mostly unmethylated in the human genome, even when inactive. and that weak CpG island promoters are distinct because they are preferential targets for de novo methylation in somatic cells [26]. According to the standard for promoters' classification in this paper, Ki-67 promoter belongs to strong CpG island promoters.

In summary, we have identified for the first time the hypomethylation status of Ki-67 promoter in vitro and in vivo. Moreover, we have provided novel insights into Ki-67 function study. Further studies are sought to explore the underline mechanism responsible for hypomethylation patterns of Ki-67 gene and to investigate the mechanisms involved in Ki-67 gene regulation at multiple levels.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Junnian Zheng, Jiangsu Key Laboratory of Biological Cancer Therapy, Xuzhou Medical College; Center of Clinical Oncology, Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, Jiangsu, PR China. Tel: +86 516 8558 6530; Fax: +86 516 8558 6530; E-mail: jnzheng@xzmc.edu.cn

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Table S1. Clinic pathological features of the patients and methylation status of Ki-67 promoter among the patients

Parameters	n	Ki-67 methylation	
		Methylated	Unmethylated
Grender			
Male	17	0	17
Female	7	0	7
Age (years)			
≥ 60	15	0	15
<60	9	0	9
Location			
Right	10	0	10
Left	12	0	12
Bilateral	2	0	2
TNM stage			
pT1	16	0	16
pT2	2	0	2
pT3	4	0	4
pT4	0	0	0
Nuclear grade			
G1	8	0	8
G2	11	0	11
G3	5	0	5

Table S2. Primers designed for different kinds of PCR

Purpose	Sequences	Amplicon size (bp)
Ki-67 RT-PCR	Sense: 5'-aattcagactccatgtgcctgag-3'	150
	Anti-sense: 5'-cttgacacacacattgtcctcagc-3'	
hTERT RT-PCR	Sense: 5'-tctaccggaagagtgtctggagcaa-3'	202
	Anti-sense: 5'-gctcccacgacgtagtccatgttca-3'	
β-actin RT-PCR	Sense: 5'-agcgagcatcccccaaagtt-3'	285
	Anti-sense: 5'-gggcacgaaggctcatcatt-3'	
Bisulfite-sequencing	Sense (P1): 5'-ttggttttttaagaaaaggatagtagg-3'	319
	Anti-sense (P2): 5'-ccctccacttccttcttccaac-3'	
	Sense (P3): 5'-ggttggaagaaggaagtggagg-3'	368
	Anti-sense (P4): 5'-cccgaacaactcccaaaaatc-3'	
MSP	Sense (M1): 5'-attcgattcggtgggagtcgtt-3'	209
	Anti-sense (M2): 5'-gcaaaaaaaaccgacgccg-3'	
	Sense (U1): 5'-tgatttggtgggagttgttagagttg-3'	210
	Anti-sense (U2): 5'-caccacaaaaaaaaccaacaccac-3'	
Promoter cloning	Sense (K1): 5'-ttgtctcgagatgcgtgagtggctcgcc-3'	223
	Anti-sense (K2): 5'-tgtgaagcttccgcccgcagcgtcagccc-3'	
	Sense (K1): 5'-ttgtctcgagatgcgtgagtggctcgcc-3'	994
	Anti-sense (K3): 5'-tgtg aagctt tcgaccccgctcct-3'	