Regulation of DEK expression by AP-2α and methylation level of DEK promoter in hepatocellular carcinoma

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Abstract. DEK is overexpressed in multiple invasive tumors. However, the transcriptional regulatory mechanism of *DEK* remains unclear. In the present study, progressive-type truncation assay indicated that CpG2-2 (-167 bp/+35 bp) was the *DEK* core promoter, whose methylation inhibited DEK expression. Bisulfite genomic sequencing analysis indicated that the methylation levels of the *DEK* promoter in normal hepatic cells and tissues were higher than those in hepatocellular carcinoma (HCC) cells. TFSEARCH result revealed transcription factor binding sites in CpG2-2. Among the sites, the AP-2α binding site showed the most significant methylation difference; hence, AP-2α is a key transcription factor that regulates DEK expression. Point or deletion mutation of the AP-2α binding site significantly reduced the promoter activity. Chromatin immunoprecipitation assay demonstrated the binding of AP-2α to the core promoter. Furthermore, knock down of endogenous AP-2α downregulated DEK expression, whereas overexpression of AP-2α upregulated DEK expression. Thus, AP-2α is an important transcription factor of DEK expression, which is correlated with the methylation level of the *DEK* core promoter in HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide; this disease ranks fifth in global morbidity and is the third primary cause of cancer-related mortality (1). Approximately 700,000 new liver cancer cases and deaths are reported annually worldwide, half of which occur in China (2). HCC accounts for 70-85% of total liver cancer cases (3). Most patients with HCC are already in middle and advanced stages when properly diagnosed because of lack of effective diagnostic approaches; although HCC could be treated by excision, therapeutic approaches for this disease are limited, resulting in poor prognosis. Hence, novel specific biomarkers for early diagnosis and prognosis evaluation of HCC must be developed.

Human DEK is a highly abundant chromatin architectural protein (4-7). This protein was initially found in the DEK-CAN fusion protein in acute myeloid leukemia (AML) (8,9). Many studies showed that the DEK protein significantly influences cell cycle and participates in multiple cell functions, such as maintaining the integrity of heterochromatin (10), transcriptional regulation (11), mRNA splicing (12), DNA replication (13) and damage repair and susceptibility (14). The DEK protein is also related to cell apoptosis (15,16). The relationship between *DEK* and tumor has been increasingly investigated. Studies showed that DEK is overexpressed in multiple malignant human tumors, such as bladder (17), colorectal (18) and gastric cancer (19). In particular, DEK is upregulated in primary HCCs compared with that in matched nonmalignant liver tissues. Thus, DEK is related to poor prognosis and could be used as an independent tumor biomarker for predicting the prognosis of tumors. *DEK* is also correlated with the occurrence and development of multiple malignant human tumors. However, only few studies reported the overexpression regulatory mechanism of *DEK* in tumors, particularly the transcriptional regulatory mechanism of *DEK* in HCC.

DNA methylation has been extensively studied in epigenetics. Researchers reported the presence of abnormal DNA methylation during the formation and development of multiple tumors. DNA methylation occurs when the methyl group ($-CH_3$) is transferred from S-adenosylmethionine to the 5' cytosine of the DNA sequence under the catalysis of DNA methyltransferases (20). Methylation in the genome of mammals mainly occurs at the CpG site. The CpG sequence is not uniformly distributed in the genome; the sequence appears less frequently in most regions than other dinucleotide sequences but is more frequent in some regions. A CpG island is a region with high cytosine and guanine contents, sequence length >200-500 bp, GC content >50% and CpG proportion >0.6 (21). The CpG island mainly exists in the gene promoter region and near the first exon region. Among normal human genomes, the CpG island in genes frequently expressed in cells, such as housekeeping genes, is under non-methylated state. Moreover, inactive genes are normally under high-methylated

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Table I. Primer sequences for luciferase reporter gene vector construction.

Name	Primer sequences (5'-3')	Length (bp)
CpG1	F-CTAGCTAGCAAAGCATCTGCATAGATGACCTAG R-CCCAAGCTTCCTGGAAAAGATGATGAGCAGTC	428
CpG2-1	F-CTAGCTAGCTCCAGGAAGCGACCGTGGAAACAATAAAC R-CCCAAGCTT TTCAAAATGGCGGTTCGGGAAGGAG	345
CpG2-2	F-CTAGCTAGCACTCCAGGCGCAGCCGGGGAGA R-CCCAAGCTTTTTCAAAATGGCGGTTCGGGAAGGAG	202
CpG3	F-CTAGCTAGCAGCATCTGCATAGATGACCTAGAACTC R-CCCAAGCTTGCTCCCCAGAATCAACAAGATTTTC	787

state, which inhibits their expression. CpG sites on non-CpG islands are under methylated state, which could be preserved stably through cell division (22).

CpG island in tumor cells manifests methylation abnormality. CpG island methylation in promoter regions is related to transcriptional silencing; conversely, CpG site methylation beyond the promoter region is called genosome methylation, which is related to transcriptional activation. Low methylation levels of the entire genome and high methylation levels of the CpG island in a local region occur simultaneously during tumor formation (23). This abnormal methylation is mainly presented as follows. High methylation levels in the promoter region results in the silencing of cancer suppressor gene, whereas low methylation levels of some oncogenes results in the activation of gene transcription. Given that *DEK* is a candidate biomarker for tumor diagnosis, the relationship between its overexpression and promoter methylation level, as well as transcriptional regulation of the *DEK* gene, requires further study to elucidate the regulatory mechanism of *DEK* overexpression in HCC. The present study aims to provide additional basis for conducting *DEK* research on HCC treatment.

Materials and methods

Cell culture. Human HCC cell lines and normal hepatic L02 cells were obtained from the American Type Culture Collection and the Chinese Academy of Sciences, respectively. The cells were cultured in Dulbecco's modified Eagle's medium (DMED; Gibco-Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2 at 37˚C. Normal hepatic tissues were supplied by the PLA General Hospital after receiving informed consent from the patients and obtaining the approval of hospital authorities. The tissues were stored in liquid nitrogen. The institutional ethics committee approved this study.

Western blot analysis. Cells were harvested by trypsinization, counted, and ultrasonically lysed with 200 μl of 1X sodium dodecyl sulfate (SDS) loading buffer per $1x10^6$ cells. Protein samples were prepared and loaded at 20 μl per lane for SDS-PAGE. The samples were electrotransferred onto nitrocellulose filter membrane, and non-specific binding was blocked in Tris-buffered saline with Tween buffer containing 5% non-fat dried milk (Yili). Western blot analysis was performed with DEK and AP-2α (16448-1-AP and 13019-3-AP; Proteintech) antibodies, followed by anti-rabbit IgG conjugated with horseradish peroxidase. GAPDH (TA-08; ZSGB-Bio, Beijing, China) was used as loading control. Chemiluminescence signals were quantified using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Real-time quantitative PCR. Total RNA was isolated from cultured cells by using the SV Total RNA Isolation system of RNA extraction kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Total RNA (1 μg) was used to synthesize cDNAs by using GoScript™ Reverse Transcription system (Promega). RT-qPCR reactions using SuperReal PreMix Plus (SYBR-Green; Tiangen Biotech Co., Beijing, China) were performed using Roche LightCycler 480 System. The expression of the gene of interest was normalized to that of *B2M*, and relative expression was calculated using comparative Ct method. PCR primers were as follows: *DEK*, forward, 5'-CAGGCACTGTGTCCTCAT-3' and reverse, 5'-CATTTG GTTCGCTTAGCCT-3'; *B2M*, forward, 5'-GGCTATCCAGC GTACTCC-3' and reverse, 5'-ACGGCAGGCATACTCA TC-3'.

Reporter gene constructs. Genomic DNA was extracted from eight cell lines and normal hepatic tissues by using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. *DEK* promoter CpG island region was truncated into four fragments, namely, CpG1 (-732 bp/-305 bp), CpG2-1 (-310 bp/+35 bp), CpG2-2 (-167 bp/+35 bp) and CpG3 (-730 bp/+57 bp). Four pairs of primers for PCR amplification were designed by Primer Premier 5.0 software (Table I). The amplification products were cloned into the pGL3-Basic luciferase reporter gene vector (Promega) and named as pGL3-*DEK*/CpG1, pGL3-*DEK*/CpG2-1, pGL3-*DEK*/CpG2-2 and pGL3-*DEK*/CpG3. The linear plasmids digested by *Hin*dIII of the four recombinant plasmids were treated with *Sss*I methylation enzyme, and their methylation status was identified using BstUI (methylation-sensitive restriction enzyme). The completely methylated linear plasmids were digested by *Nhe*I to obtain the full methylation of the four target fragments, which were cloned into the pGL3-Basic vector. The resulting vectors were called pGL3-Basic/CpG1 (Me), pGL3-Basic/CpG2-1 (Me), pGL3-Basic/CpG2-2 (Me) and pGL3-Basic/CpG3 (Me).

Table II. Primer sequences for BSP.

Name	Primer sequences (5'-3')	Length (bp)
C1	F-TTTTATATTTATAGGGGTGTAAATTTATGT	294
	R-CTTCCTAAAAAAAATAATAAACAATCCC	
C2	F-GGGATTGTTTATTATTTTTTTTAGGAAG	381
	R-CCAAAATCAACAAAATTTTCAAAATAAC	

Transfection and luciferase assays. The cells were plated in 96-well or 24-well plates, grown to 60-80% confluence, and transfected with small-interfering RNA (siRNA) or plasmids by using Lipofectamine® 2000 reagent (Invitrogen) according to the manufacturer's instructions. Cells transfected with luciferase constructs were harvested and lysed in Passive lysis buffer (Promega) after 48 h. The cell lysates were analyzed for firefly and *Renilla* luciferase activity by using the Dual-Luciferase reporter assay system (Promega) as recommended by the manufacturer. Firefly luciferase activity was normalized to *Renilla* luciferase activity as internal control.

Bisulfite genomic sequencing. The genomic DNA of eight HCC cell lines and normal hepatic tissue was treated with sodium bisulfite by EpiTect® Fast Bisulfite Conversion DNA kit (Qiagen). PCR amplification fragment should not be too long (<500 bp) because the treated genomic DNA becomes unstable and easy to break. Thus, we divided the CpG island sequence into two short segments to amplify 294 and 381 bp in the *DEK* promoter -595 bp/-302 bp and -329 bp/+52 bp, respectively. The corresponding segments were named C1 and C2. We then designed two pairs of primers (Table II) for the treated C1 and C2 for bisulfite sequencing PCR (BSP). BSP was performed in 40 cycles of 98˚C for 10 sec, 55˚C for 30 sec, and 72˚C for 30 sec. Finally, the PCR products were cloned into the pMD18-T vector (Takara). At least 10 clones were randomly selected for bisulfite genome sequencing (BGS) to analyze the methylation status of the *DEK* promoter CpG island.

Prediction of transcription factor binding sites of DEK promoter CpG island region. Bioinformatics methods were used to predict the CpG island location in the *DEK* promoter region near the sequence from -1000 to +400 bp range by MethPrimer online software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). The transcription factor binding sites of *DEK* promoter CpG island region were predicted by TFSEARCH online software (http://www.cbrc.jp/research/db/TFSEARCH.html).

Point and deletion mutation assays. We analyzed point and deletion mutation of the binding site 'GCCCGCGGC' of the transcription factor AP-2α in the CpG2-2 region. Point mutation is the substitution of the underlined consensus sequence 'GCCCGCGGC' into 'TAACGCGGC'. Deletion mutation includes the deletion of the underlined part of 'GCCCGCGGC' and the removal of all 'GCCCGCGGC'. The three kinds of mutational CpG2-2 fragments, namely, pGL3-basic/PM, pGL3-basic/FM and pGL3-basic/AM, were cloned into the

pGL3-Basic vector (Promega). Finally, HepG2 cells were transfected with the plasmids by using Lipofectamine® 2000 reagent (Invitrogen) according to the manufacturer's instructions. Blank plasmid pGL3-basic and pGL3-*DEK*/CpG2-2 were used as negative and positive controls. Luciferase activity was assayed after 24 h.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was performed with EpiQuik ChIP kit (P-2002-1; EpiGentek, Farmingdale, NY, USA) following the manufacturer's instructions. A total of 1x10⁶ HepG2 cells were fixed in 1% formaldehyde at room temperature for 10 min. The cells were then lysed and chromatin was sheared by sonication. The DNA-protein complex was immunoprecipitated with anti-RNA Pol II antibody (P-2002-1; EpiGentek), anti-IgG antibody (P-2002-1; EpiGentek), and anti-AP-2α antibody (13019-3-AP; Proteintech). After reverse cross-linking and DNA purification, DNA from input (1:20 diluted) or immunoprecipitated samples were assayed by PCR. The products were separated by 3% agarose gel electrophoresis. The primers for the *DEK* promoter were (forward) 5'-GCGACCGTGGAAACAATAAACA-3' and (reverse) 5'-TGCCTCCGCGGGAAGCTC-3'. Primers for the positive control *GAPDH* were (forward) 5'-ACGTAGCTCAGGCCTCAAGA-3' and (reverse) 5'-GCGGGCTCAATTTATAGAAAC-3' (P-2002-1; EpiGentek).

Overexpression and small-interfering RNA interference assays of transcription factor AP-2α. AP-2α expression plasmid was constructed according to the CDS coding region of TFAP-2α (NM_001032280.2) in the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/). The modified CDS of TFAP-2α was inserted with the His tag sequence 'CATCACCATCACCATCAC' behind the transcription start site 'ATG' and added with two restriction enzyme cutting sites on both ends, namely, 5'-end *Eco*RI 'GAATTC' and 3'-end *Hin*dIII 'TTCGAA'. The modified CDS was then cloned into the pcDNA3.1 (-) vector (Invitrogen) and named pcDNA3.1 (-)-hAP2. The vector was transfected into HepG2 cells, and the expression levels of *AP-2α* and *DEK* were assayed. In addition, *AP-2α* siRNA interference sequences and siRNA-negative control (NC) (Table III) used in this study were designed and synthesized by GenePharma. Equimolar amounts of three oligonucleotides specific for *AP-2α* were combined before transfecting 7721 cells. Blank control, NC, and the experiment group were set up for the transfection experiment. Total RNA was extracted using SV Total RNA Isolation system of RNA extraction kit (Promega) after 24 h of transfection. Total protein was extracted after transfection of 48 h. Finally, the mRNA and

Table III. *AP-2α* small-interfering RNA interference sequences.

Name	Sense (5'-3')	Antisense (5'-3')
siRNA-1	CCAGAUCAAACUGUAAUUAdTdT	UAAUUACAGUUUGAUCUGGdTdT
siRNA-2	GGAAGAUCUUUAAGAGAAAdTdT	UUUCUCUUAAAGAUCUUCCdTdT
siRNA-3	CCUGCUCACAUCACUAGUAdTdT	UACUAGUGAUGUGAGCAGGdTdT
siRNA-NC	UUCUCCGAACGUGUCACGUdTdT	ACGUGACACGUUCGGAGAAdTdT

Table IV. Primer sequences for reverse transcription-PCR.

Name	Primer sequences (5'-3')	Length (bp)
DEK	F-CAGGCACTGTGTCCTCAT R-CATTTGGTTCGCTTAGCCT	316
AP-2α	F-CGTCTCCGCCATCCCTATTA R-GGTTTCGCACACGTACCCAA	364
B2M	F-GGCTATCCAGCGTACTCC R-ACGGCAGGCATACTCATC	247

protein expression levels of *AP-2α* and *DEK* were investigated by RT-PCR and western blot analyses. The primer sequences for *AP-2α* and *DEK* are shown in Table IV.

Statistical analysis. Statistical analysis was performed using SPSS software. Results were analyzed by Student's t-test, and differences were considered statistically significant at P<0.05. All *in vitro* experiments were performed in triplicate, and the average result is reported. Error bars depict standard error.

Results

DEK is overexpressed in HCC. Fluorescent quantitative PCR and western blot analyses were performed to determine the mRNA and protein expression levels of DEK in HCC cell lines, respectively. The results showed that *DEK* mRNA and protein were overexpressed in all HCC cell lines compared with those in normal hepatic cell line L02 and tissues (Fig. 1). Gel-Pro Analyzer was used to quantify the western blotting results, and two-tailed Student's t-test indicated that *DEK* mRNA and protein expression levels in the seven HCC cell lines (P<0.05 or P<0.01) significantly differed from those in L02 and normal hepatic tissues. Among the cell lines, 7402 and 7721 HCC cells exhibited the highest DEK expression level. DEK was found to be overexpressed in HCC.

CpG2-2 (-167 bp/+35 bp) is the core promoter region of DEK. Sequences near the *DEK* promoter region within -1000 bp/+400 bp were analyzed through bioinformatics methods with the MethPrimer online software. Potential CpG island was located within -436 bp/+345 bp (Fig. 2A). The extension of the *DEK* core promoter was then identified. A series of reporter gene constructs was obtained by progressive-type truncation strategy, transfected into HepG2 cells, and then analyzed with the Dual-Luciferase reporter assay system. The results
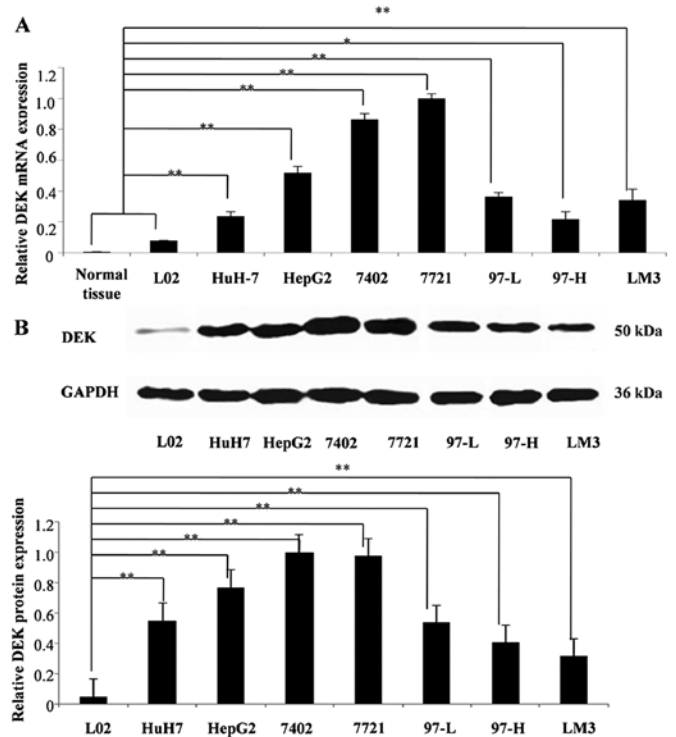

Figure 1. Analysis of DEK expression level by real-time quantitative PCR (RT-qPCR) and western blot analyses. (A) *DEK* mRNA level in hepatocellular carcinoma (HCC) cell lines was assayed by RT-qPCR, with normal hepatic cell L02 and normal tissues as controls. (B) DEK protein level was detected by western blotting and gray scanning analysis of Gel-Pro Analyzer software, with normal hepatic cell L02 as control. Data are shown as averages of three independent experiments. *P<0.05, **P<0.01.

demonstrated that among the constructs without methylation *in vitro*, the activities of pGL3-*DEK*/CpG3, pGL3-*DEK*/CpG2-1, and pGL3-*DEK*/CpG2-2 were higher than those of pGL3-*DEK*/CpG1 compared with the controls (P<0.01). However, the activities of pGL3-*DEK*/CpG2-1 and pGL3-*DEK*/CpG2-2 were not significantly different from that of pGL3-*DEK*/CpG3 (P>0.05). Further analysis showed that pGL3-*DEK*/CpG2-2 reserved most of the activity of pGL3-*DEK*/CpG2-1 (P>0.05) (Fig. 2B); hence, CpG2-2 (-167 bp/+35 bp) is the minimum core promoter region of the *DEK* gene. In addition, no activity was found in HepG2 cells for constructs methylated *in vitro* compared with that in NC. This result illustrates that hypermethylation of the *DEK* promoter inhibited its activity in HCC.

DEK promoter in HCC is commonly under low-methylation state. BSP and BGS were performed using genomic DNA of eight HCC cell lines and normal hepatic tissues to investigate
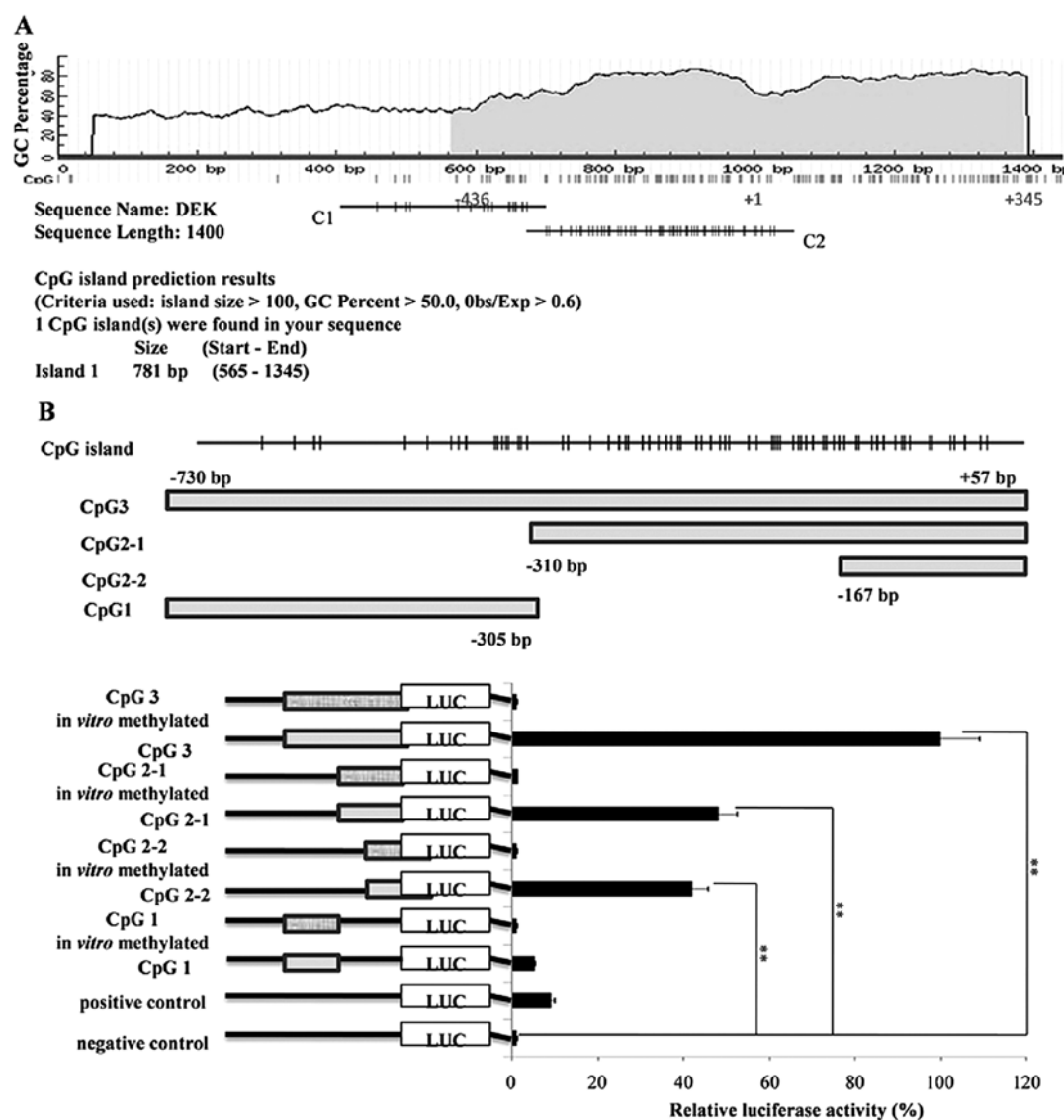
Figure 2. Identification of the core promoter of human *DEK* in HepG2 cells and effect of DNA methylation on promoter activity. (A) The CpG island of *DEK* promoter region near the sequence from -1000 bp to +400 bp range predicted by MethPrimer online software was located in -436 bp/+345 bp region. In the schematic structure of the CpG island, short vertical lines represent CpG sites, and '+1' denotes transcription start site. (B) Identification of the core promoter of human *DEK* by truncation analysis. CpG island region was truncated into four fragments, namely, CpG1 (-732 bp/-305 bp), CpG2-1 (-310 bp/+35 bp), CpG2-2 (-167 bp/+35 bp), and CpG3 (-730 bp/+57 bp). HepG2 cells were transiently transfected with the luciferase reporter constructs. Reporter gene activity was assayed by the Dual-Luciferase reporter assay system, and pGL3-basic vector was used as negative control. Relative firefly luciferase activity was calculated as the average of three independent transfections normalized to *Renilla* control activity (**P<0.01).

whether the methylation level of the *DEK* promoter is related to its overexpression in HCC. We encoded the sequencing results into QUMA online software for comparative analysis. The atlas shown in Fig. 3A illustrates the detailed CpG methylation sites of *DEK* promoters. The results proved that the methylation levels of *DEK* promoters in normal hepatic tissues and normal hepatic L02 were significantly higher than those in the HCC cell lines; moreover, methylation sites were mostly located in the CpG2-2 region. We then calculated the total ratio of methylation sites and plotted the methylation ratio histogram (Fig. 3B) to investigate the methylation level of *DEK* promoters in all cell lines and tissues. The result showed that DEK promoters in HCC were commonly under low-methylation state compared with those in normal cells and tissues; hence, *DEK* overexpression in HCC was correlated with the low-methylation level of the promoters.

Transcription factor binding sites of DEK core promoter prediction. We speculated that the *DEK* core promoter region may contain important transcription factor binding sites that play pivotal roles in maintaining the basal transcriptional activity of *DEK*. Hence, we analyzed the core promoter region CpG2-2 (-167 bp/+35 bp) by using TFSEARCH online software. The results showed that DNA binding sites for four transcription factors, namely, nuclear factor Y (NF-Y), AP-2α, E2F and Ying Yang 1 (YY1), were found in the CpG2-2 region. According to the BGS results (Fig. 3A), transcription factor binding sites containing CpG methylation sites in normal hepatic cells and tissues were screened; these sites included AP-2α, E2F and YY1 (Fig. 4A). Among these sites, the binding site for AP-2α presented the most significant methylation difference compared with that in HCC and was located in sites no. 45 and 46 CpG (Fig. 3A). The results suggested
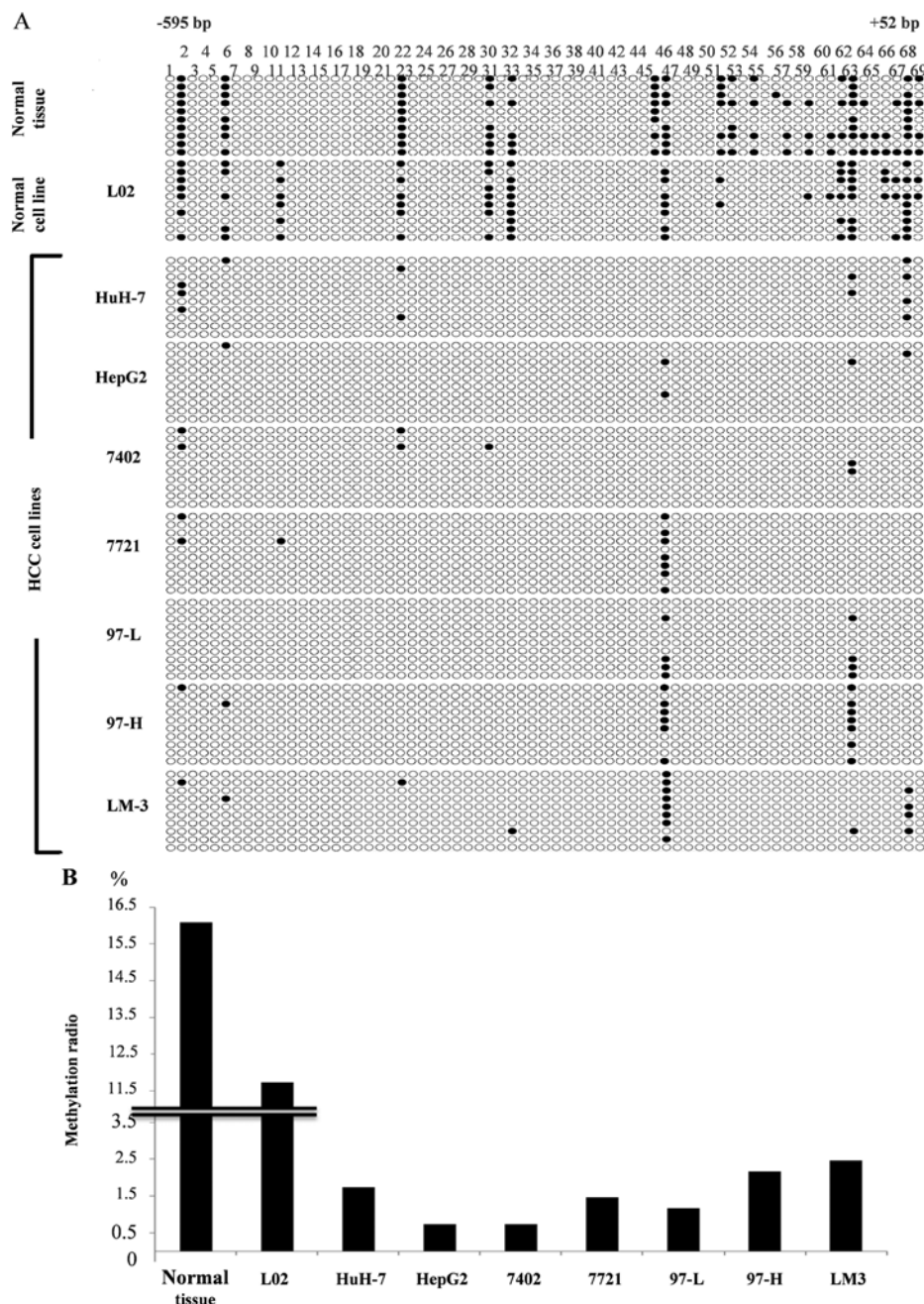
Figure 3. Methylation analysis of *DEK* promoter in HCC cells, L02 and normal hepatic tissues. (A) Representative high-resolution analysis of the methylation status of individual CpG sites in the *DEK* promoter (C1 and C2, -595 bp/+52 bp) by bisulfite genomic sequencing in HCC cell lines and normal hepatic cell L02 and tissues. At least 10 clones were randomly chosen and sequenced. Each circle corresponds to a single CpG site. Methylated sites are indicated by filled dark circles, whereas unmethylated sites by empty white ones (●, methylatedCpG site; ○, unmethylatedCpG site) from -595 bp to +52 bp in turn numbering 1, 2, 3,...68 and 69. (B) The methylation ratio of every cell and tissue was calculated, and methylation ratio histogram was plotted.

that *DEK* overexpression in HCC was correlated with the demethylation of the AP-2α transcription factor binding site. In addition, transcription factors NF-Y, E2F and YY1 were previously reported to regulate DEK expression (24,25). We will focus on the regulation effect of the transcription factor AP-2α for DEK expression in further studies.

AP-2α binding site plays a dominant role in maintaining DEK core promoter activity. We evaluated the effect of the predicted AP-2α transcription factor binding site on DEK core promoter activity. We performed point and deletion mutations in the wild-type plasmid pGL3-*DEK*/CpG2-2 and transfected the constructs into HepG2 cells. Luciferase activities were determined after 24 h. The results showed that *DEK* core promoter activity was significantly reduced after point and deletion mutations of the AP-2α transcription factor binding site (P<0.01). In particular, all deletion mutation-type pGL3-basic/AM led to ~60% reduction in DEK core promoter activity compared with that in the wild-type pGL3-*DEK*/CpG2-2 (Fig. 4B). This result indicated that the AP-2α transcription factor binding site plays a dominant role in maintaining the transcription activity of the *DEK* core promoter. We also predict that the newly found transcription factor AP-2α may significantly regulate DEK expression.

A

-167 ACTCCAGGCG CAGCCGGGGA GAGCCCCGCG CGCGCATCCC TCC**GCCCGCG**
                                                      AP-2

-117 **GC**CGAGGCGA CCCCTCGCGG TCCCGCCCGC CCGAGC**TTCC CGC**GGAGGCA
                                              E2F

-67 CCCGGCCGCC CCGCCTCCCC CGCCCCGCGC CCGCCAACTC CCAGCTCGCG

-17 GCCCTGATTG GTCGCCGCA**T TCCCGC**TCTC CTTCCCGAAC **CGCCATTTT**G
                        E2F   YY1
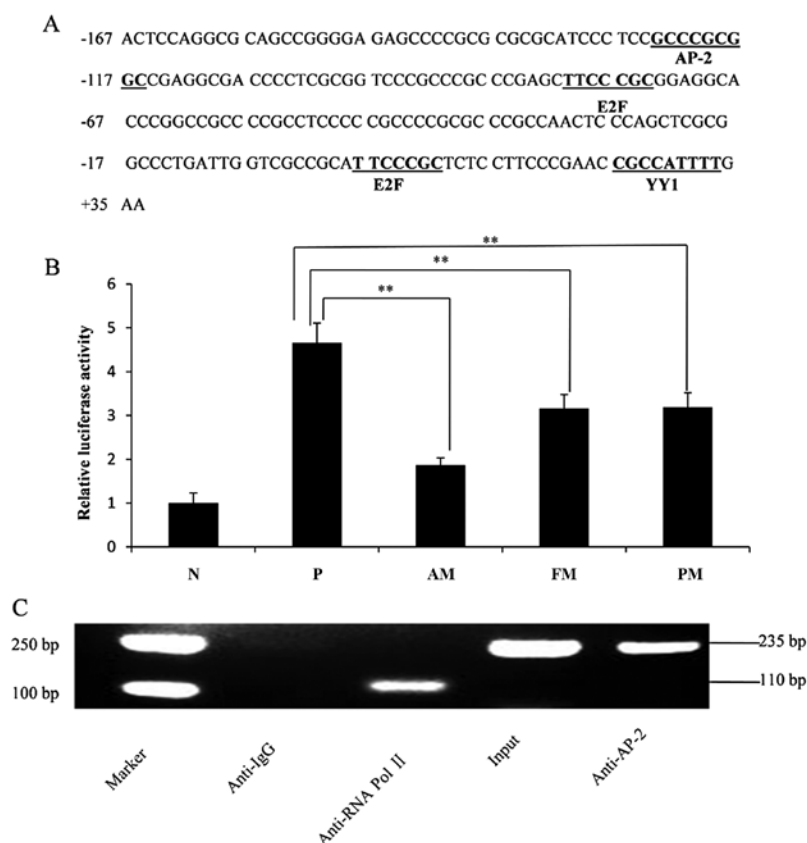
+35 AA

B

C

Figure 4. Characterization of transcription factor binding sites. (A) Nucleotide sequence of the *DEK* core promoter was located from -167 bp to +35 bp. Putative transcription factor binding sites were underlined. (B) Mutation analysis of putative transcription factor AP-2α binding site. HepG2 cells were transfected with the pGL3-basic vector, the wild plasmid pGL3-*DEK*/CpG2-2, three nucleotide substitution mutant variant (pGL3-basic/PM), six site-deletion mutant variant (pGL3-basic/FM), and all site-deletion mutant variant (pGL3-basic/AM). Luciferase activity was measured after 24 h. The negative control was normalized to 1, and others were compared with the normalized level. N, negative control; P, positive control; PM, point mutation; FM, fraction deletion; AM, all deletion. Transfections were performed in triplicate for each experiment (**P<0.01). (C) The binding of AP-2α to the DEK core promoter *in vivo* using Chromatin immunoprecipitation assay. The nucleoprotein complex from HepG2 cells was immunoprecipitated with anti-IgG, anti-RNA Pol II and anti-AP-2α antibodies, respectively. Then, the precipitated and purified DNA fragments were subjected to PCR. Anti-IgG antibody (negative control) failed to precipitate any protein-DNA complex *in vivo*; anti-RNA Pol II antibody (positive control) precipitated the RNA Pol II bound to the GAPDH promoter and the PCR amplified products were 110 bp (RNA Pol II is considered to be enriched in the *GAPDH* gene promoter); anti-AP-2α antibody precipitated AP-2α proteins bound to the *DEK* core promoter which contains the predicted AP-2α binding sites 'GCCCGCGGC' *in vivo* and its PCR amplified products were 235 bp. The input served as the internal reference and measure the efficiency of ChIP.

Transcription factor AP-2α binds to the DEK core promoter in vivo. AP-2α is a transcription factor that binds to the promoter regions of its target genes, which contain the binding sites 'GCCCGCGGC', and mediates the transcription of the target genes. We performed ChIP assay to validate whether AP-2α interacts with the *DEK* core promoter *in vivo*. The nucleoprotein complex prepared from HepG2 cells was immunoprecipitated with anti-RNA Pol II, anti-IgG and anti-AP-2α antibodies. The precipitated DNA was subjected to PCR with primers flanking the region containing the AP-2α binding site. The PCR results showed that anti-AP-2α antibody precipitated proteins were bound *in vivo* to the amplified sequence of the DEK promoter; by contrast, non-specific IgG antibody (NC antibody) failed to precipitate proteins bound *in vivo* in this sequence (Fig. 4C). Thus, the transcription factor AP-2α could bind to the *DEK* core promoter region *in vivo*.

Transcription factor AP-2α upregulates the expression level of the DEK gene. AP-2α siRNA or AP-2α expression vector pcDNA3.1 (-)-hAP2 was transfected into 7721 or HepG2 cells to determine the role of AP-2α in regulating DEK expression. *DEK* mRNA and protein levels were detected by RT-PCR and western blot analyses, respectively. The results illustrated that siRNA mediated 53% knockdown of AP-2α mRNA and 80% knockdown of the *AP-2α* protein, resulting in reduction in *DEK* mRNA level by 51% and DEK protein level by 63% (Fig. 5A and B). By contrast, overexpression of *AP-2α* mRNA by 1.9-fold and AP-2α protein by 2.0-fold led to 2.5-fold increase in *DEK* mRNA level and a substantial increase of 90% in the DEK protein level compared with those in the blank control (Fig. 5C and D). Thus, we conclude that the expression of DEK was mediated by the transcription factor AP-2α.

Discussion

We performed RT-qPCR and western blot analyses to verify that DEK was really overexpressed in HCC. The mRNA and protein expression levels of DEK increased, which were identical with the results reported in previous studies (26-28). However, minimal information is available on the regulation of DEK expression. The overexpression of DEK in HCC was
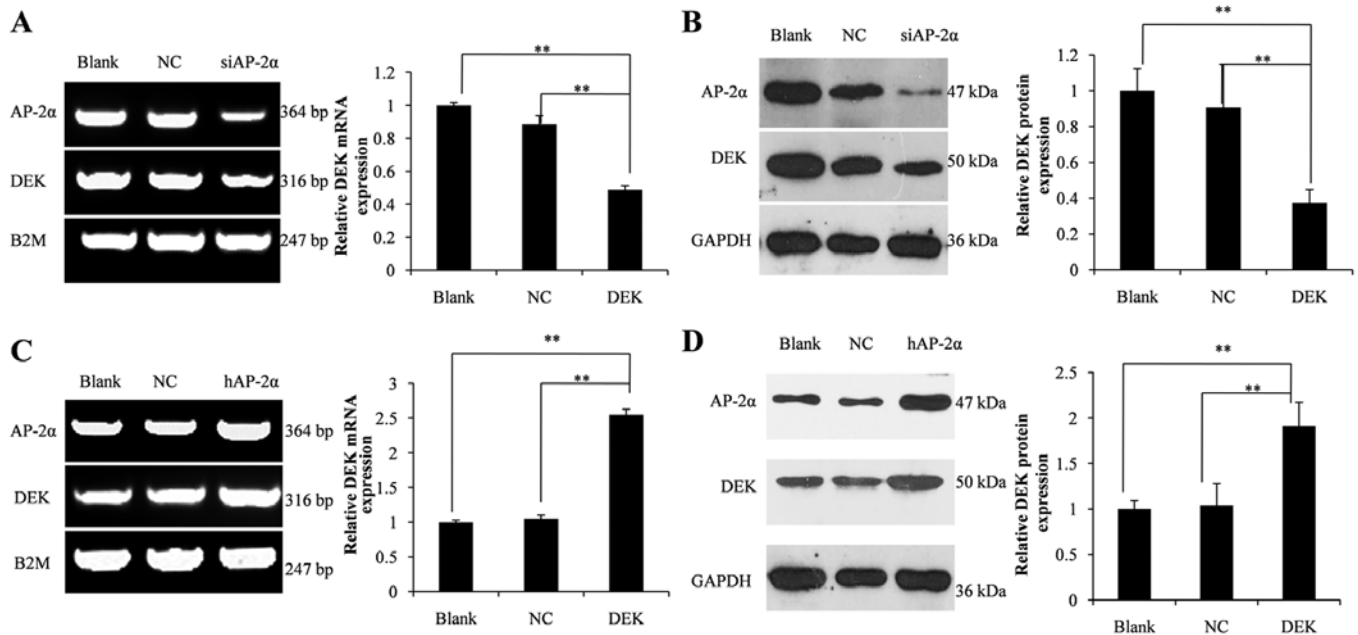
Figure 5. AP-2α regulation of DEK expression in HCC cells. HepG2 cells were transiently transfected with AP-2α siRNA and negative control siRNA. A total of 7721 cells were transiently transfected with pcDNA3.1 (-) empty vector and pcDNA3.1 (-)-hAP2. (A and C) *DEK* mRNA was determined through RT-PCR 24 h after transfection, and the gray scanning results of BandScan were normalized to B2M. Data are representative of three independent experiments (**P<0.01). (B and D) Interference efficiency, overexpression efficiency, and DEK protein expression were analyzed by western blotting with anti-AP-2α, anti-DEK and anti-GAPDH antibodies. Gray scanning results of BandScan were normalized to GAPDH. The blank control was normalized to 1, and others were compared relative to the normalized level. Blank, blank control; NC, negative control. Data are representative of three independent experiments (**P<0.01).

initially reported to be of S-phase dependency and could exert carcinogenic effect by inhibiting p53 activity (29). Previous studies showed that the proportion of *DEK* mRNA overexpression in HCC tissues was rather high, which may be related to the conversion of normal hepatic tissues into HCC (26). In addition, reports suggested that *DEK*, as an independent risk factor in HCC, promoted tumor invasiveness, resulting in the poor prognosis of HCC (27). Hence, the abnormal overexpression of DEK in tumors would probably become a biomarker for early diagnosis or vicious transformation. Nevertheless, studies on the transcriptional regulatory mechanism of the abnormal overexpression of DEK in tumors were not comprehensive. The *DEK* promoter contains transcription factor binding sites bound by transcription factors E2F, YY1, ERα, NF-Y and c-myc. E2F and YY1 were bound to the sequences near the *DEK* transcription start site, which induced the formation of a transcriptional initiation complex to activate the relevant passageways and boost tumor formation.

Several studies on the transcriptional regulatory mechanism of genes in tumors revealed that decreased methylation levels of oncogenes are common in human tumors. Methylation level is inversely correlated with gene expression. The low-methylation level of many genes in tumor tissues occurs in the promoter region. The low-methylation level of LINE-1 in chronic granulocytic leukemia could initiate its sense transcription and antisense transcription of *c-MET* (30). Numerous activated and amplified *c-fos* genes were found in ovarian cancer tissues of mice, primarily because of the low-methylation level of the *c-fos* gene (31). Proto-oncogene *c-myc* was also under the low-methylation state in tumor tissues, which led to significant upregulation of gene expression and then accelerated cell malignant proliferation, resulting in

tumor occurrence (32). Additional studies reported that the promoter region of estrogen receptor (ER) regulatory gene *pS2* in breast cancer was lowly methylated, which is significantly related to the upregulation of ER (33). Moreover, the low-methylation level of multiple genes often occurs in the same kind of tumor. For example, genes, such as *claudin 4*, *LCN2*, *TFF2*, *S100A4* and *mesothelin*, are all lowly methylated in pancreatic cancer (34).

The present study revealed that the functional minimal core promoter CpG2-2 is located within the 202 bp region at position -167 bp/+35 bp relative to the transcription start site by Dual-Luciferase reporter assay. BGS was performed to evaluate the effect of methylation level on *DEK* promoter transcriptional activity. The results suggested that *DEK* promoter in HCC cell lines was commonly under low-methylation state compared with normal hepatic tissues and normal hepatic cell line L02. Moreover, methylation sites focused on CpG2-2 region, which indicated that DEK overexpression in HCC is related to the low-methylation level of the core promoter. Hypermethylation exerted a remarkable inhibiting effect on its transcriptional activity. According to the BGS results, AP-2α transcription factor binding site predicted by TFSEARCH online software was screened as the most hypermethylated site in normal hepatic cells and tissues. Furthermore, we verified whether transcription factor AP-2α regulates DEK expression. We performed point and deletion mutations of the AP-2α DNA binding site and ChIP assays. The results demonstrated that the AP-2α binding site was crucial for the transcription activity of the *DEK* core promoter, and AP-2α transcription factor could bind to the *DEK* core promoter region *in vivo*. Knocking down endogenous AP-2α led to reduction in DEK expression. Conversely, overexpression of AP-2α upregulated

DEK expression. The transcription factor AP-2α regulates DEK expression by directly binding to the AP-2α binding site.

In summary, the present study reveals that the *DEK* core promoter is located in the -167 bp/+35 bp region. DEK overexpression in HCC is regulated by the transcription factor AP-2α and closely correlated with the methylation level of the core promoter. This study further reveals the transcriptional regulatory mechanism of DEK overexpression in HCC and provides additional basis for early diagnosis, prognosis judgment, and genetic therapy in HCC.

Acknowledgements

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