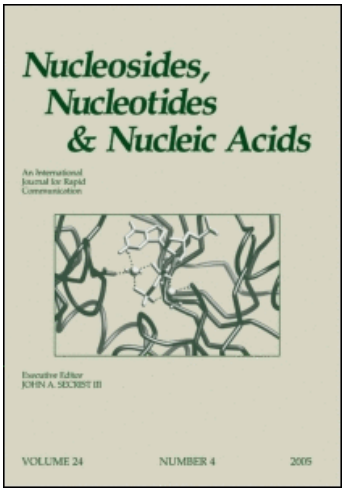

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METHYLATION SPECIFIC PCR TO CHARACTERIZE METHYLATION OF THE PROMOTER OF DEOXYCYTIDINE KINASE

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□ *Deoxycytidine kinase (dCK) is essential for phosphorylation of natural deoxynucleosides and analogs, such as gemcitabine and cytarabine, two widely used anticancer compounds. We hypothesized that DNA methylation of SP1 binding sites in the dCK promoter region might affect dCK expression. Using methylation specific PCR (MSP), methylation was detected in one of the SP1 binding sites of the dCK promoter, in most tested cancer cell lines and in patient samples from brain tumors and leukemia. This SP1 site is a 3′ GC box, which upon hypomethylation negatively regulates dCK mRNA expression. In conclusion, we developed a new MSP method showing methylation of the 3′ GC-box in the dCK promoter region in tumor cells and patient samples. Methylation might therefore regulate transcription of dCK, and should be studied further to understand its role in influencing gemcitabine and cytarabine activity.*

Keywords Gemcitabine; deoxycytidine kinase; methylation specific PCR; promoter methylation

INTRODUCTION

Deoxycytidine kinase (dCK) catalyzes the phosphorylation of several deoxynucleosides and their analogs such as gemcitabine (dFdC) and cytarabine (ara-C),[1,2] which are widely used for the treatment of childhood and adult leukemia, pancreatic and non-small cell lung cancer. Deficiency of dCK has been linked to resistance to these drugs,[3,4] possibly due to mutations and splice variants, and the antitumor effect of gemcitabine is related to dCK

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activity.[5] The dCK gene is composed of 7 exons, is localized on chromosome 4,[6–8] consists of a homodimer of 60 kD,[7] is expressed throughout the cell cycle,[9] but shows variable levels in different tissues.

The dCK promoter has four CpG-rich regions, representing four potential SP1 binding sites, an E2F binding site, and an E-box (USF binding site).[7] One of these SP1 sites is a 3′-GC transcription inhibition box.[10] Limited data addressing the DNA methylation status of dCK indicated that epigenetic silencing was not a common feature of acute lymphoblastoid leukemia cell lines that were resistant to Ara-C,[11] while no methylation was observed within −267 to +155 bp regarding to the transcription start site in hepatocellular carcinoma[12] nor within the 0.3 kb 5′ region, enclosing exon 1 and intron 1.[10] We hypothesized that epigenetic regulation in particular DNA methylation of SP1 binding sites could be responsible for dCK regulation in cancer cells from various origins and gemcitabine resistant cell lines. Therefore we designed a methylation specific PCR (MSP) method for the detection of possible methylation in the promoter of the dCK gene.

MATERIALS AND METHODS

Leukemic and solid tumor cell lines were cultured under standard conditions in DMEM (solid tumor cells) or RPMI (leukemia) medium with 10% FCS and 0.1% glutamine. Patient samples (AML, brain tumors and normal bone marrow) were obtained from the VU University Medical Center Amsterdam. The study was approved by the institution's ethical review board.

DNA was isolated from cell pellets containing 5–10 × 10^6 cells using Trizol as described earlier.[13] The amount and purity of DNA was determined using a Nanodrop instrument. For detection of methylation in the promoter of dCK we developed a MSP method. MSP was preceded by bisulfite treatment, which converted unmethylated cytosine to uracil, while methylcytosine is resistant for this reaction. This bisulfite reaction was performed as described earlier.[13] Primers for the MSPs were designed based on the four potential SP1 binding sites in the promoter of the dCK gene. For every site a methylated and an unmethylated primer set was manufactured (Isogen, De Meern, the Netherlands). All primers were between 15 and 30 basepairs in length and were called dCK1–dCK4; dCK4 is a 3′GC box (Table 1). The MSP reaction was performed as described earlier.[13] The extent of methylation can only be judged qualitatively by this method, providing the same amount of DNA that was used for the assay. Methylation was considered negative (−) when no band was present, and positive (+), when a band was present (Figure 1 and Table 2).

TABLE 1 Details of the MSP primers

Primer set	SP1 location on dCK promoter	Sense primer 5′ → 3′	Antisense primer 5′→ 3′	Product length (bp)	Anneal temp °C
dCK1 M	233–243	tttatttttttttttattcgatttcg	atcacgtaaaaaccgaaacg	114	52.4
dCK1 U		ggtttatttttttttttttatttgattttg	acaaatcacataaaaaccaaaaca	120	52.4
dCK2 M	292–299	gttggaggcgggcg	aatacgcacactaaaactcgc	201	66.4
dCK2 U		aatttgtgttggaggtgggtg	accacaaatacacacactaaaactcaca	215	66.3
dCK3 M	333–345	gaggagggcggggtc	gcacactaaaactcgcgacg	151	58.8
dCK3 U		tttgaggagggtggggtt	cacaaatacacacactaaaactcacaaca	163	58.8
dCK4 M	388–396	ggtagttagggagcgcg	ctaataaactcaccgacccg	120	57.6
dCK4 U		gagtgtgtagtgggaatttgtg	caactaataaactcaccaaccca	130	57.6

The dCK promoter region was labeled according to.[7]

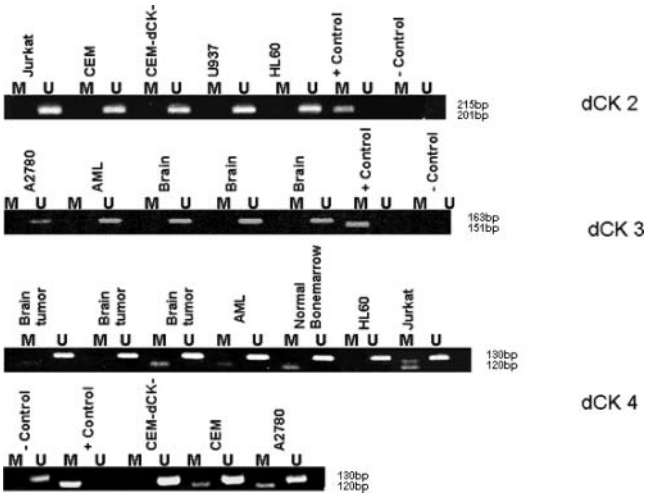

FIGURE 1 Representative agarose gels showing methylation patterns in various cell lines and patient samples for the dCK2, dCK3 and dCK4 primers (SP4). For dCK4 the band for the unmethylated primers (U) has 130 bp, that for the methylated primers (M) 120 bp. The positive control is in vitro methylated and bisulfite converted DNA, the negative control is human placenta which is normally unmethylated.

RESULTS

MSP was performed with all primer sets on leukemic and solid tumor cell lines, and on patient samples of brain tumors, AML and normal bone marrow. In all MSP analyses, the positive control (in vitro methylated and bisulfite

TABLE 2 Summary of all cell lines and patient samples subjected to MSP

		dCK1		dCK2		dCK3		dCK4 (3′-GC)	
Cell line	Origin	M	U	M	U	M	U	M	U
Jurkat	Leukemia	−	+	−	+	−	+	+	+
CEM	Leukemia	−	+	−	+	−	+	+	+
CEM-dCK-	Leukemia	−	+	−	+	−	+	−	+
U937	Leukemia	−	+	−	+	−	+	−	+
HL60	Leukemia	−	+	−	+	−	+	−	+
MV4–11	Leukemia	−	+	−	+	−	+	−	+
Kasumi-1	Leukemia	−	+	−	+	−	+	−	+
A2780	Ovarian cancer	−	+	−	+	−	+	+	+
AG6000 (dCK-)	Ovarian cancer	−	+	−	+	−	+	-	+
HEK	Human embryonic Kidney	−	+	−	+	−	+	+	+
	Bone marrow	−	+	−	+	−	+	+	+
	Acute myeloid leukemia	−	+	−	+	−	+	+	+
	Brain tumor #1	−	+	−	+	−	+	+	+
	Brain tumor #2	−	+	−	+	−	+	−	+
	Brain tumor #3	−	+	−	+	−	+	+	+

−, no methylation; +, methylation present. dCK1, dCK2, CK3, dCK4 are potential methylation sites; dCK4 covers the 3′GC box; M, primer set for methylated DNA, U, primer set for unmethylated DNA. dCK-, dCK deficient cells. The unmethylated band was always present (+).

converted DNA) produced a band with the primers specific for methylated DNA (M-primers), but not with the negative control (human placenta which is normally unmethylated) while primers specific for unmethylated DNA (U-primers) bound to bisulfite converted DNA and as expected did not bind to in vitro methylated DNA. dCK1, dCK2, and dCK3 all produced bands with the unmethylated primer sets on all cell lines and patient samples (Table 2). With the dCK4 primers the M-band and the U-band were visible in 3 cell lines, while the M-band was absent in 6 other samples (Figure 1; Table 2). In the patient samples strong U-bands were detected, while the M-bands were faint (Figure 1).

DISCUSSION

Methylation of the dCK promoter has been demonstrated at the 3′-GC box transcription inhibition box of various cancer cell lines. Several other parts of the promoter have been investigated earlier,[11,14,15] but no methylation was observed. The goal of this study was to set up MSP assays for detection of methylation in the promoter of the dCK gene in various forms of cancer. We therefore chose 4 CpG rich SP1 binding sites in the promoter of the dCK gene which may be important in gene transcription.[7] The first three binding sites did not show methylation. The primers specific for the fourth SP1 binding site (dCK4) encoding a 3′-GC box, produced bands with both the methylated and the unmethylated set. This site is a 3′-GC activation inhibition box and a negative regulator of dCK promoter activity.[10] Future studies should address the biological significance of methylation at this site, for example, on dCK expression, drug sensitivity and modulation by azacytidine.

In conclusion, we have developed a new MSP method showing methylation of a 3′ GC-box in the dCK promoter in tumor cells and patient samples. This methylation might regulate dCK transcription, possibly leading to a higher activity, and our MSP will allow screening of patients treated with gemcitabine and cytarabine, in order to evaluate whether this methylation can affect drug activity.

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