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Expression of deoxycytidine kinase in leukaemic cells compared with solid tumour cell lines, liver metastases and normal liver

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Abstract

Deoxycytidine kinase (dCK) is required for the phosphorylation of several deoxyribonucleoside analogues that are widely employed as chemotherapeutic agents. Examples include cytosine arabinoside (Ara-C) and 2-chlorodeoxyadenosine (CdA) in the treatment of acute myeloid leukaemia (AML) and gemcitabine to treat solid tumours. In this study, expression of *dCK* mRNA was measured by a competitive template reverse transcriptase polymerase chain reaction (CT RT-PCR) in seven cell lines of different histological origin, 16 childhood and adult AML samples, 10 human liver samples and 11 human liver metastases of colorectal cancer origin. The enzyme activity and protein expression levels of dCK in the cell lines were closely related to the mRNA expression levels ($r=0.75$, $P=0.026$ and $r=0.86$, $P=0.007$). In AML samples, *dCK* mRNA expression ranged from 1.16 to 35.25 ($\times 10^{-3} \times dCK/\beta\text{-}actin$). In the cell line panel, the range was 2.97–56.9 ($\times 10^{-3} \times dCK/\beta\text{-}actin$) of *dCK* mRNA expression. The enzyme activity in liver metastases was correlated to *dCK* mRNA expression ($r=0.497$, $P=0.05$). In the liver samples, these were not correlated. *dCK* mRNA expression showed only a 36-fold range in liver while a 150-fold range was observed in the liver metastases. In addition, dCK activity and mean mRNA levels were 2.5-fold higher in the metastases than in the liver samples. Since dCK is associated with the sensitivity to deoxynucleoside analogues and because of the good correlation between the different dCK measurements in malignant cells and tumours, the CT-RT PCR assay will be useful in the selection of patients that can be treated with deoxycytidine analogues.

Keywords: Deoxycytidine kinase; Solid tumours; AML; Gemcitabine; Ara-C

1. Introduction

Deoxycytidine kinase (dCK) (EC 2.7.1.74) is a pyrimidine salvage enzyme that phosphorylates deoxycytidine, deoxyadenosine and deoxyguanosine. Furthermore, it is responsible for the phosphorylation of several deoxynucleoside analogues, which are widely used as anticancer and antiviral agents, such as 1-β-D-arabinosyl cytosine (cytarabine, Ara-C), 2′2′-difluorodeoxycytidine (gemcitabine, dFdC) and 2-chlorodeoxyadenosine (cladribine, CdA). The activity of dCK shows a wide variation in normal and malignant cells and tissues [1,2].

The level of dCK activity has been reported to be closely related to sensitivity to deoxynucleoside analogues such as CdA [3], Ara-C and dFdC [4–9]. A deficiency of dCK leads to a resistance to deoxycytidine and deoxyadenosine analogues [10,11], therefore pretreatment measurements of dCK might be of predictive value [5]. However, relative large cell numbers or tumour biopsy specimens are required for measurements of activity. The use of the polymerase–chain reaction (PCR) enables us to use small biopsy specimens or a small number of cells. Therefore, we applied reverse transcriptase (RT)-PCR techniques to develop a sensitive assay for the expression of *dCK* mRNA. In order to quantify the expression, we used competitive templates

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(CTs) for *dCK* mRNA which were included in the same reaction mixture.

In this study, *dCK* mRNA expression was compared with dCK activity and protein expression in a panel of solid tumour and leukaemia cell lines, as well as in samples from adult and paediatric leukaemia patients, and from liver and liver metastases from patients with colorectal cancer.

2. Materials and methods

2.1. Materials

[8-^{3}H]-2-Chloro-2′-deoxyadenosine (24.2 Ci/mmol) was obtained from Moravek, Brea, CA, USA. RNAzol was purchased from Campro Scientific (Veenendaal, The Netherlands); Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) and RNase inhibitor (25 IU/μl) were obtained from Promega (Madison, WI, USA). Deoxyribonucleotides (dNTPs), random hexamers, and Taq polymerase (5 IU/μl) were purchased from Amersham/Pharmacia Biotech (Roosendaal, The Netherlands). Primers were from Isogen (Maarssen, The Netherlands). RESponse Research agarose was obtained from Biozym (Landgraaf, The Netherlands). The rabbit anti-human dCK polyclonal antibody was produced and tested at the Institute of Molecular Biology and Biotechnology at Crete by the group of Dr I. Talianidis [12].

2.2. Cells

The cancer cell lines BxPC3 (pancreas), UMSCC14C (head and neck) A2780 (ovarian), H322 (non-small cell lung), CCRF CEM (T-cell acute lymphoid leukaemia), U937 (histiocytic lymphoma) and HL60 (acute myeloid leukaemia (AML)) were used to measure dCK expression and activity. The variants AG6000 (A2780 resistant to gemcitabine), CCRF CEM-dCK$^-$ (CCRF CEM resistant to AraC) were included to set the lowest level of dCK activity. The solid tumour cell lines were cultured in Dulbecco's modification of Eagles medium (Bio Whittaker Europe, Verviers, Belgium) supplemented with 5% fetal calf serum (FCS) (Gibco, Paisly, UK). The others were cultured in Roswell Park Memorial Institute (RPMI) medium (Bio Whittaker) with 10% FCS. Subconfluent flasks with cells were harvested and counted. The cell pellets were frozen at −70 °C until further analysis.

2.3. Acute myeloid leukaemia samples

Mononuclear blast cells were derived after informed consent from 4 adult and 12 paediatric patients with acute myeloid leukaemia as previously described in Ref. [13] and isolated by density gradient methods. Contaminating non-leukaemic cells were removed by immunomagnetic beads resulting in a percentage of leukaemic cells above 80%, as morphologically determined by May–Grünwald–Giemsa staining of the cytospins.

2.4. Liver and liver metastases

Biopsies of liver metastasis and normal liver of patients with histologically-proven colorectal cancer were used to study dCK in solid tumours and normal tissue. Samples were immediately frozen in liquid nitrogen and subsequently stored at −80 °C. Frozen tissues were pulverised using a microdismembrator [1], powdered tissue could be stored for the different dCK assays.

2.5. Deoxycytidine kinase activity

dCK activity in each of the cell lines was determined using ^{3}H-CdA as a substrate, since CdA is a better and more specific substrate than deoxycytidine, which can also be phosphorylated by thymidine kinase 2. The assay was based on a method described by Arnér and colleagues in Ref. [14]. Cell pellets (at least 1.5×10^7) were resuspended in 50 mM Tris(hydroxymethyl)-aminomethane (TRIS)/HCl buffer (pH 7.4), containing 4 mM dithiothreitol (DTT), at a concentration of 30×10^6 cells/ml and kept on ice. Frozen tissue was suspended in 50 mM TRIS/HCI buffer (0.33 g/ml). Cells were disrupted by sonification and the suspension was centrifuged for 15 min at 7000*g*. The supernatant was used for the enzyme assay and protein determination. We used 25 μl of supernatant (6–126 μg protein) and added 10 μl 50 mM MgATP/100 mM NaF, 10 μl 250 μM [8-^{3}H]-CdA (specific activity 0.19 μCi/nmol) and 5 μl TRIS/HCl buffer. This mixture was incubated at 37 °C. The assay was stopped by heating the samples to 90 °C, then 10 μl of 10 mM CdA was added to enable its localisation by ultraviolet (UV) absorbance on the PEI thin layer sheets. The samples were centrifuged in order to spin down the protein and subsequently 5 μl was spotted onto polyethyleneimine (PEI) cellulose thin-layer chromatography sheets. The sheets were developed with water to separate substrate and product. The different spots were cut and put into a vial with 750 μl of 0.1 M HCl/0.2 M KCl to enable elution of radioactivity from the PEI cellulose, during 2 h of shaking. After addition of liquid scintillation fluid, radioactivity was counted. Enzyme activity was expressed as pmol CdA-MP formed per h per mg protein.

2.6. Competitive template RT-PCR

The isolation of RNA was performed with RNAzol as previously described in Ref. [13]. Cell pellets were

suspended in an aliquot of 200 μl of RNAzol per 10^6 cells. Twenty micrograms of the isolated RNA was used for reverse transcription into cDNA. Random hexamers were used as primers for M-MLV-RT at a concentration of 0.045 μg/μl. After a brief incubation at 56 °C to remove secondary structures, samples were quickly cooled down on ice and annealing of the hexamers also took place on ice. The extension of the primers was at 42 °C using M-MLV-RT. The reaction was terminated by heating at 95 °C for 5 min. cDNA samples were stored at −20 °C until further use.

The design of the primers and the CT was based on the method described by Willey and colleagues in Refs. [13,15,16]. One forward (A) and two reverse primers (BC and C) were selected by Oligo software requiring an optimal annealing temperature of 58 °C, absence of hairpins and no predictable stable primer-dimer formations.

A: 5′-GAAGGGAACATCGCTGCAGG
C: 5′-TGGCCAAATTGGTTATTCATCC
BC: 5′-TGGCCAAATTGGTTATTCATCCTTGAG-CTTGCCATTCAGAGAGG

The primers A and C were used to amplify a native template of 425 bp covering exons 2, 3 and part of 4 of *dCK*. The primers A and BC were selected for the construction of the CT. The B sequence is upstream of C and amplification with A and BC resulted in a shorter product of 294 bp. This product was the CT linked by the A and C sequence, which was further purified and quantified as described in Refs. [13,15]. The *β-actin* gene was used as a reference gene and the previously published construction of the *β-actin* CT was used [16].

CTs of *β-actin* and *dCK* were mixed in a ratio of 10, 100 and 1000 (10^{-12} M/10^{-13} M; 10^{-12} M/10^{-14} M; 10^{-12} M/10^{-15} M) based on preliminary experiments. One single master mix was prepared for every cDNA sample containing PCR buffer (1×), $MgCl_2$ (1.5 mM), dNTPs (200 μM), Taq polymerase (5 μl, final concentration 0.02 U/μl), sample cDNA and CT mix in a total volume of 46 μl. *dCK* and *β-actin* primers (4 μl, final concentration 4 ng/μl of each primer) were added to different tubes, which already contained certain aliquots of the master mix. Reaction mixtures were overlayed with mineral oil and cycled in a MJ Research PTC-200 (Biozym, Landgraaf, The Netherlands). A semi-hot start from ice to 94 °C was performed and with 1-min steps of denaturation at 94 °C, primer annealing at 58 °C and elongation at 72 °C the PCR reaction continued for 35 cycles.

PCR products were separated by electrophoresis on 2% agarose gels containing 0.1 μg/ml ethidium bromide. Quantification was by digital image analysis using Scion Software. The concentration of native template molecules in the cDNA samples was calculated by the ratio of the intensity of native template/CT and the molarity of the CT mixture as previously described in Refs. [13,15,16]. Normalisation of the *dCK* expression to the expression of *β-actin* was based on data obtained from assays with the same master mixture.

The *dCK* expression in the cell lines was determined at least three times on different cDNA samples of one cell line. The expression in the AML samples was measured once, because these samples were also used for several other assays. Levels of expression were reported as units *dCK* mRNA *β-actin* mRNA $\times 10^{-3}$ molecules.

2.7. Western blotting

Western blots were produced according to a published method in Ref. [12]. Protein extracts (30 μg) were electrophoresed on 12.5% sodium dodecyl sulphate (SDS)-polyacrylamide gels and transferred by electroblotting to nitrocellulose membranes (Hybond ECL membranes, Amersham, Buckinghamshire, UK). After overnight incubation in blocking buffer (5% bovine serum albumin in TRIS-buffered saline with 0.1% Tween 20), the blot was probed with rabbit anti-human dCK polyclonal antibody (1:5000, 1 h at room temperature) and goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:40,000, 1 h at room temperature). Purified recombinant dCK (10 ng), extracts of AG6000 cells lacking dCK and parental A2780 ovarian cancer cells were tested on every blot to identify the dCK band. Levels of relative expression were determined by densitometric scanning (Imaging Densitometer, model GS-690, Biorad of the X-ray films (Hyperfilm ECL). dCK protein levels were expressed as ng dCK per mg total protein.

2.8. Statistics

The Spearman correlation test was used to quantify the relationship between dCK activity and dCK expression at the mRNA and protein level. Student's *t*-test was used for comparison of paired and unpaired samples.

3. Results

The panel of cell lines had a 10-fold variation in dCK activity. The lower level was set by CCRF-CEM/dCK$^-$ and AG6000 (1.2 and 1.4 pmol/h per mg protein, respectively). The mRNA expression showed a 19-fold variation for the cell lines (Table 1). No *dCK* mRNA could be detected in CCRF-CEM/dCK$^-$. AG6000 showed a normal and a truncated mRNA product as has been previously published [6], this complicated accurate quantification and this cell line was excluded from the mRNA expression evaluation (Fig. 1). The reproducibility of the CT-RT-PCR assay in the cell lines is given in Table 1 as the relative standard deviation.

Table 1
dCK mRNA expression in cell lines and AML samples[a]

Sample Cell line	*dCK* mRNA expression Ratio $10^{-3}\times dCK/\beta\text{-}actin$	Relative standard deviation (%)
CCRF CEM	38.0±17.3	33
HL60	56.3±19.9	26
U937	56.9±6.60	7
A2780	6.06±6.76	15
UMSCC14C	4.73±0.38	10
BxPC3	2.97±0.16	9
H322	5.97±1.54	6
AML		
0438*	4.24	
4695*	1.87	
4779	3.12	
6250*	10.79	
7470	26.30	
7578	35.23	
7621	19.40	
7747	1.16	
7985	2.77	
8019	12.27	
8051	9.88	
8069	3.52	
8196	26.17	
8400*	11.39	
8773	3.00	
8774	4.40	

AML, acute myeloid leukaemia.

[a] mRNA expression is the mean ± standard error of the mean (S.E.M.) of at least three measurements on different samples for the cell lines. The relative standard deviation (S.D.) is based on 2–3 measurements on the same sample. (*) Indicates adult AML samples, the others are from childhood AML; not enough clinical material was available to measure enzyme activity. No expression was measured in the Ara-C- and gemcitabine-resistant cell lines.

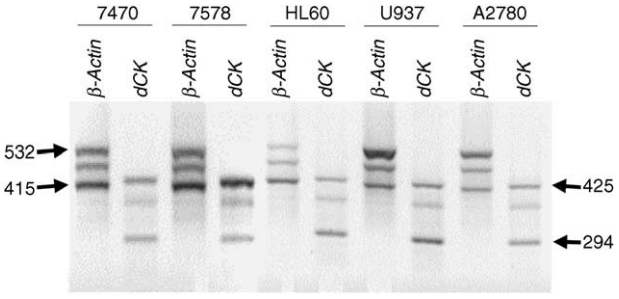

Fig. 1. Representative X-ray photos of *β-actin* and *dCK* gene and competitive template (CT) products of the cell lines and 2 AML samples (7470 and 7578). The blots shows three bands for both *dCK* and *β-actin*: 532 and 425 are the number of the basepairs encoded by the forward and reverse primers for the native cDNA of *β-actin* and *dCK*, respectively; 415 and 294 are the number of basepairs encoded by the competitive templates for *β-actin* and *dCK*, respectively. The intermediate band is a heterodimer (HD) which can be formed between the native cDNA and the competitive template. The contribution of the HD was calculated as previously described in Refs. [13,16]. The bands were scanned and the optical density (OD) was used to calculate a ratio between the native cDNA and CT of each target, therafter these ratios were used to calculate the ratio for *dCK* and *β-actin*.

The AML samples had a 30-fold range of *dCK* mRNA expression that overlapped with that of the cell lines, although the high level of expression in the cell lines HL60 and U937 was not seen in the patient samples.

Liver metastases contained a mean 2.5-fold higher dCK enzyme activity than the surrounding normal liver samples (10.1±1.9 and 4.0±0.4 nmol/h/mg protein, respectively, $P=0.004$, Student's *t*-test for paired data) (Fig. 2). The range of dCK activities in the liver metastases was about 17-fold (1.4–23.8 nmol/h per protein), while that in the liver ranged only 3-fold (2.0–6.7). The *dCK* mRNA expression showed a wide variation for these samples: a 150-fold difference (0.53–80.4). The subgroup of liver samples had a 36-fold range (0.63–22.7) in *dCK* mRNA expression levels.

The correlation between dCK activity and *dCK* mRNA levels in the cell lines is depicted in Fig. 3a. The correlation coefficient determined by Spearman rank correlation was $r=0.75$ ($P=0.026$). The correlation between

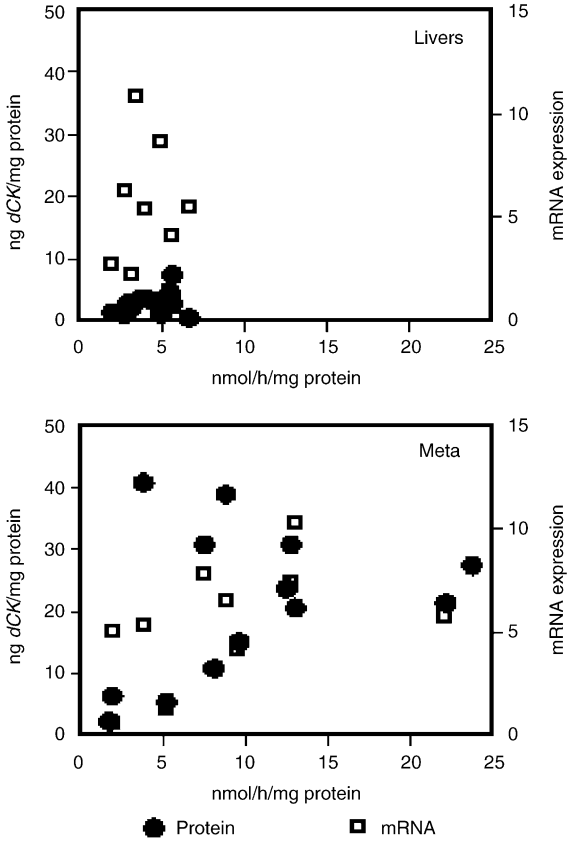

Fig. 2. Activity of dCK, amount of dCK protein and *dCK* mRNA expression in normal livers and liver metastases (meta) from colorectal cancer. The dCK activity in liver metastases was significantly higher than in livers ($P=0.004$). In livers, no correlation was found between dCK activity and *dCK* mRNA, while dCK activity was related to dCK protein ($r=0.59$; $P=0.013$). However, in liver metastases, dCK activity was correlated with *dCK* mRNA ($r=0.497$; $P=0.05$), but not to dCK protein. One liver metastasis sample is not shown having 72.9 ng dCK/mg protein and 80.4 as *dCK* mRNA expression. One liver sample had a *dCK* mRNA of 22.7 and a dCK activity of 5 nmol/h/mg protein.

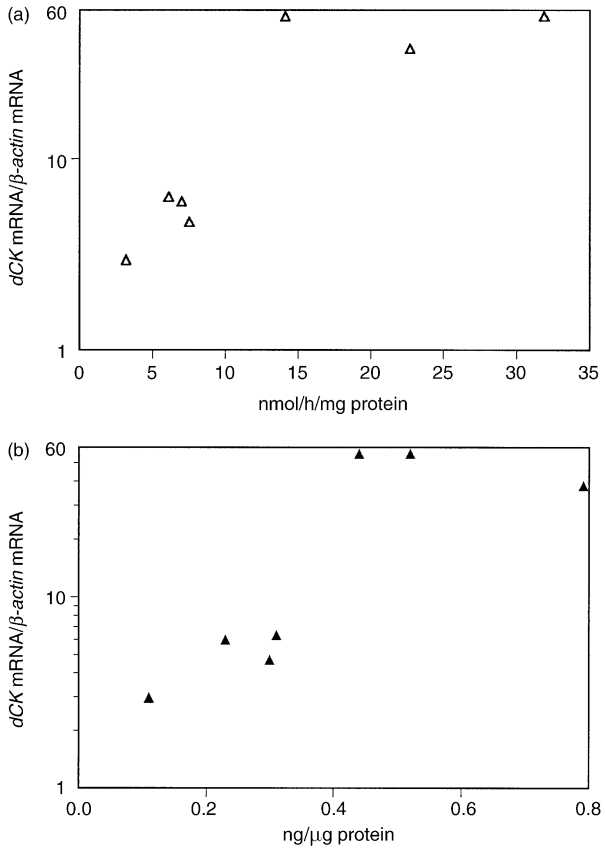

Fig. 3. (a) Correlation between mRNA expression and dCK activity in a panel of seven cell lines of different origin. Correlation coefficient $r=0.75$ ($P=0.026$). (b) Correlation between mRNA expression and dCK protein expression in a panel of seven cell lines of different origin. Correlation coefficient $r=0.79$ ($P=0.018$).

these dCK measurements in pooled liver and liver metastases is $r=0.416$ ($P=0.024$). The correlation was also observed for liver metastases only (Fig. 2) ($r=0.497$, $P=0.050$), but not for the subgroup of livers.

The dCK protein expression in the cell line panel (Figs. 3b and 4) varied about 7.2-fold. The correlation with *dCK* mRNA (Fig. 3b) was $r=0.85$ ($P=0.007$), as determined by Spearman rank correlation. In addition, the correlation coefficient of protein expression and activity was significant ($r=0.79$, $P=0.018$, Spearman).

In the liver metastases, dCK protein levels were clearly higher and had a broader range compared with the normal liver samples (5.3–72.9 and 0.5–7.4 ng dCK/mg protein, respectively) (Fig. 2). A correlation between

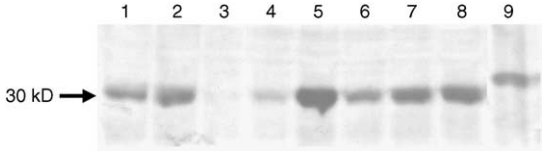

Fig. 4. Western blot of dCK protein in the different cell lines (1, 14C; 2, A2780; 3, AG6000; 4, BxPC3; 5, CEM; 6, H322; 7, HL60; 8, U937; 9, dCK-HIS).

dCK activity and protein was found in the liver samples ($r=0.59$, $P=0.013$), but not in the metastases. There was no correlation between *dCK* mRNA and protein expression in these samples.

4. Discussion

The expression of *dCK* mRNA in a panel of solid tumour cell lines and leukaemia and lymphoma cell lines was closely correlated to the activity of this enzyme. This relationship had already been established for a panel of six leukaemia cell lines by Kawasaki and colleagues in Ref. [17]. For solid tumour cells from different histological origin such a relationship had not yet been established. Any such correlation is of major interest because of the important role of some novel deoxynucleoside analogues, such as gemcitabine, in the treatment of solid tumours. Our panel is a mixture of both leukaemic and solid tumour cells, and if we considered only the four solid tumour cell lines, the relationship was weaker. The correlation between *dCK* activity and dCK mRNA expression in liver metastases was also weaker and its statistical significance was borderline ($P=0.05$).

The substrate CdA, which we used, is also a substrate for deoxyguanosine kinase (dGK), albeit with a much higher K_m (85 μM) and lower efficiency than for dCK (5 μM) [18,19]. The cell lines CCRF-CEM-dCK$^-$ and AG6000 have some CdA phosphorylating capacity, but they do not show a dCK protein band in western blotting. The activity measured here is most likely due to dGK activity, which is also present in the cell extract. The positive relationship between dCK enzyme activity and protein expression has also been observed in studies of Kawasaki [20] and Spasokoukotskaja [21]. The variation in the *dCK* mRNA expression in the AML samples (30-fold) is somewhat lower than that found in a study with 13 childhood AML samples (50-fold) [22] and 35 childhood ALL and AML samples [23] in which samples from relapsed leukaemic cells had a lower *dCK* expression. In addition, for the enzyme activities, a comparable range in dCK levels was found [24].

The relationship between dCK activity and response to treatment with deoxynucleoside analogues has been investigated in several malignancies. Hairy cell leukaemia, which is very sensitive to CdA showed both high dCK and dGK activity. The degree of total CdA phosphorylation correlated with the response to CdA treatment of both hairy cell leukaemias and chronic lymphocytic B-cell leukaemias, although other factors also seemed to be involved [3,25]. In AML, dCK activity correlated with the response to Ara-C treatment in 21 patients [26]. ALL patients more often relapsed when dCK expression was low or absent [27]. Transfection experiments with *dCK* and other deoxynucleoside kinase [28] genes has underlined the important role of

dCK in the antitumour activity of deoxynucleoside analogues. Increased expression of *dCK* gene by transfection restored and enhanced the sensitivity to deoxynucleoside analogues that are activated by dCK [8,9,29], both *in vitro* and *in vivo*.

Most of the AML samples were derived from childhood AMLs [13], which are always treated with an Ara-C-containing protocol. The large range of *dCK* mRNA expression, which overlapped with the expression of the cell lines, indicates that this disease in general has an intrinsic sensitivity to Ara-C, but that individual differences in sensitivity to Ara-C exist. Moreover, this has been reported and has prognostic significance [30,31]. Recently, a clinical protocol including Ara-C was started in which dCK expression will be evaluated prospectively.

Gemcitabine is currently being used for the treatment of many solid malignancies. Single agent gemcitabine shows little activity in colorectal cancer, but it is not clear whether this is due to a low dCK expression. Therefore, we designed this study to determine dCK expression in liver metastases. No information is currently available on the relationship between response and dCK activity in patients. In cell lines, we previously reported a relationship between gemcitabine sensitivity and dCK activity [6,7], while in xenografts [5], we also observed a relationship between gemcitabine sensitivity and dCK activity. *dCK* gene transfer enhanced the antitumour effect of gemcitabine on tumour xenografts [9], confirming the relationship between dCK and gemcitabine activity. It is remarkable that the dCK activity in the liver samples in our study showed relatively small variations and did not exceed a level of 5 nmol/h/mg protein. In contrast, protein and mRNA expression levels showed more variation. In liver metastases, both activity and mRNA varied considerably and correlated with one another. This would give an advantage for liver metastases compared with liver in selectively activating deoxynucleoside analogues in liver metastases. A high dose of gemcitabine is expected to lead to selective activation in metastases, and when given via a hepatic artery infusion would not leak to the systemic circulation as livers have a high deaminase activity. The much smaller range of dCK activity in the liver samples compared with the liver metastases also indicated that in the liver a posttranslational regulation mechanism exists, which seems to be deregulated in the liver metastases. It has been reported that dCK activity can be regulated by its phosphorylation [32,33]. dCK purified from leukaemic blasts can be phosphorylated by Protein Kinase C (PKC), resulting in a 100% increase in enzyme activity [32]. However, recombinant dCK is a poor substrate for PKC, but is effectively phosphorylated by protein kinase A, and this is not accompanied by an increase in enzyme activity [33]. An enhanced dCK activity could be eliminated by protein phosphatase treatment of lymphoid cells, supporting a secondary modification of the dCK protein [34]. In addition, the promoter for *dCK* contains several potential regulatory transcription factor sites [35,36], although it is not yet clear whether this regulation is tissue-specific or deregulated in tumours.

For the enzyme activity assays and western blotting, relatively large pieces of tissue or many cells are required, and these are not always available. The current PCR assay detects a wide range of *dCK* mRNA levels. CT-RT-PCR can be performed with any PCR equipment, and does not need the requirement of an expensive Taq man or light cycler apparatus. In addition, results from this type of assay shows a high inter- and intralaboratory agreement [37]. Measurement of *dCK* mRNA expression offers the possibility for larger-scale studies, in patients with solid tumours and leukaemia, to determine a relationship between dCK expression and response to either gemcitabine or Ara-C. This data should be followed by a prospective study to select patients for deoxynucleoside analogue treatment based on their dCK activity.

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