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DCK is frequently inactivated in acquired gemcitabine-resistant human cancer cells

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ABSTRACT

Although gemcitabine is the most effective chemotherapeutic agent against pancreatic cancer, a growing concern is that a substantial number of patients acquire gemcitabine chemoresistance. To elucidate the mechanisms of acquisition of gemcitabine resistance, we developed gemcitabine-resistant cell lines from six human cancer cell lines; three pancreatic, one gastric, one colon, and one bile duct cancer. We first analyzed gemcitabine uptake using three paired parental and gemcitabine resistant pancreatic cancer cell lines (PK-1 and RPK-1, PK-9 and RPK-9, PK-59 and RPK-59) and found that uptake of gemcitabine was rapid. However, no DNA damage was induced in resistant cells. We further examined the microarray-based expression profiles of the cells to identify genes associated with gemcitabine resistance and found a remarkable reduction in the expression of deoxycytidine kinase (*DCK*). DCK is a key enzyme that activates gemcitabine by phosphorylation. Genetic alterations and expression of *DCK* were studied in these paired parental and derived gemcitabine-resistant cell lines, and inactivating mutations were found only in gemcitabine-resistant cell lines. Furthermore, siRNA-mediated knockdown of *DCK* in the parental cell lines yielded gemcitabine resistance, and introduction of *DCK* into gemcitabine-resistant cell lines invariably restored gemcitabine sensitivities. Mutation analyses were expanded to three other different paired cell lines, DLD-1 and RDLD-1 (colon cancer cell line), MKN-28 and RMKN-28 (gastric cancer cell line), and TFK-1 and RTFK -1 (cholangiocarcinoma cell line). We found inactivating mutations in RDLD-1 and RTFK-1 and decreased expression of *DCK* in RMKN-28. These results indicate that the inactivation of *DCK* is one of the crucial mechanisms in acquisition of gemcitabine resistance.

1. Introduction

Pancreatic cancer ranks fifth among cancer-related deaths in Japan and fourth in the United States [1,2]. Pancreatic cancer, typically a ductal adenocarcinoma, is characterized by an extremely poor prognosis with a total 5 year survival rate lower than 5% [2]. Surgical resection is the sole curative treatment method to date. In patients with unresectable pancreatic cancer, the mean survival time from diagnosis has been a maximum of 4 months. Evolution of palliative treatment with gemcitabine (GEM hereinafter) has modified the subsequent prognosis providing a prolonged survival to 6 or 7 months [3,4]. Innate or acquired resistance to GEM might limit the effect of this chemotherapy.

Reportedly, mechanisms involved in acquisition of GEM resistance include limited cellular uptake or increased excretion of GEM, inactivation of intracellular GEM, and activation of anti-apoptotic pathways [5]. In order to overcome GEM resistance, it is necessary to understand the specific mechanisms causing it. To approach this problem, we have established GEM-resistant cell lines from six human cancer cell lines, including three pancreatic, one gastric, one colon, and one bile duct cancer. We then examined the pharmacokinetics of GEM and conducted comprehensive expression analyses of the GEM-resistant and corresponding parental cell lines. Surprisingly, most of the cell lines with acquired

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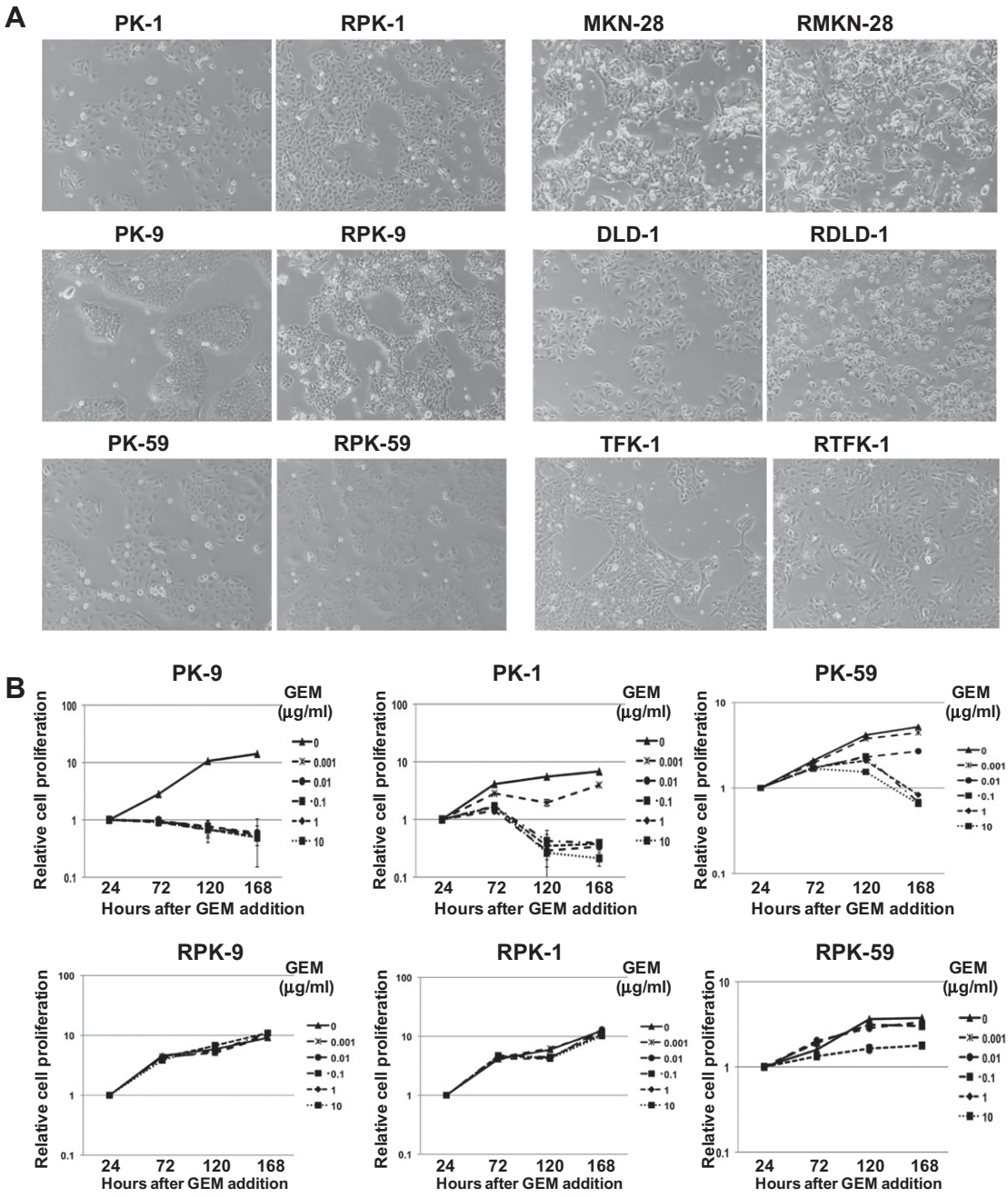

Fig. 1. (A) Morphological appearance of the parental and established resistant cancer cell lines. Magnification was x40. (B) Cell viabilities of PK-9 (relatively sensitive to GEM), PK-1 (moderately sensitive to GEM) and PK-59 (less sensitive to GEM) and their derived GEM-resistant cell lines RPK-9, RPK-1 and RPK-59, respectively, were monitored using several different GEM concentrations. These experiments were performed in quadruplicate.

GEM-resistance harbored destruction of deoxycytidine kinase (*DCK*) function; we propose that inactivating mutations of *DCK* play a crucial role in acquisition of GEM resistance.

2. Materials and methods

2.1. Establishment of GEM-resistant cell lines

We used following cancer cell lines to establish the gemcitabine hydrochloride (GEM) resistant cell lines: PK-1 [6], PK-9 [7] and PK-59 [8] (pancreatic cancer), DLD-1 [9] (colon cancer), MKN-28 [10] (gastric cancer), and TFK-1 [11] (cholangiocarcinoma). GEM was purchased from Eli Lilly (Indianapolis, IN), and GEM-resistant cancer cell lines were established by the methods previously described [12] with some modifications. In brief, each cell line at subconfluent density was exposed to GEM for more than one month at the concentration determined for individual IC_{50}. When each surviving cell colony was detected, the GEM-concentration was increased in 10-fold steps to a final concentration of 100 μg/ml. Each candidate GEM-resistant cell line was grown on GEM-free medium for more than one month and then exposed again to GEM at 100 μg/ml. If the candidate resistant cell line grew and its parental cell line could not survive, we recognized the successful establishment of acquired GEM-resistance. Established cell lines were then named with R as the prefix. From the RPK-1, RPK-9 and RPK-59 cell lines,

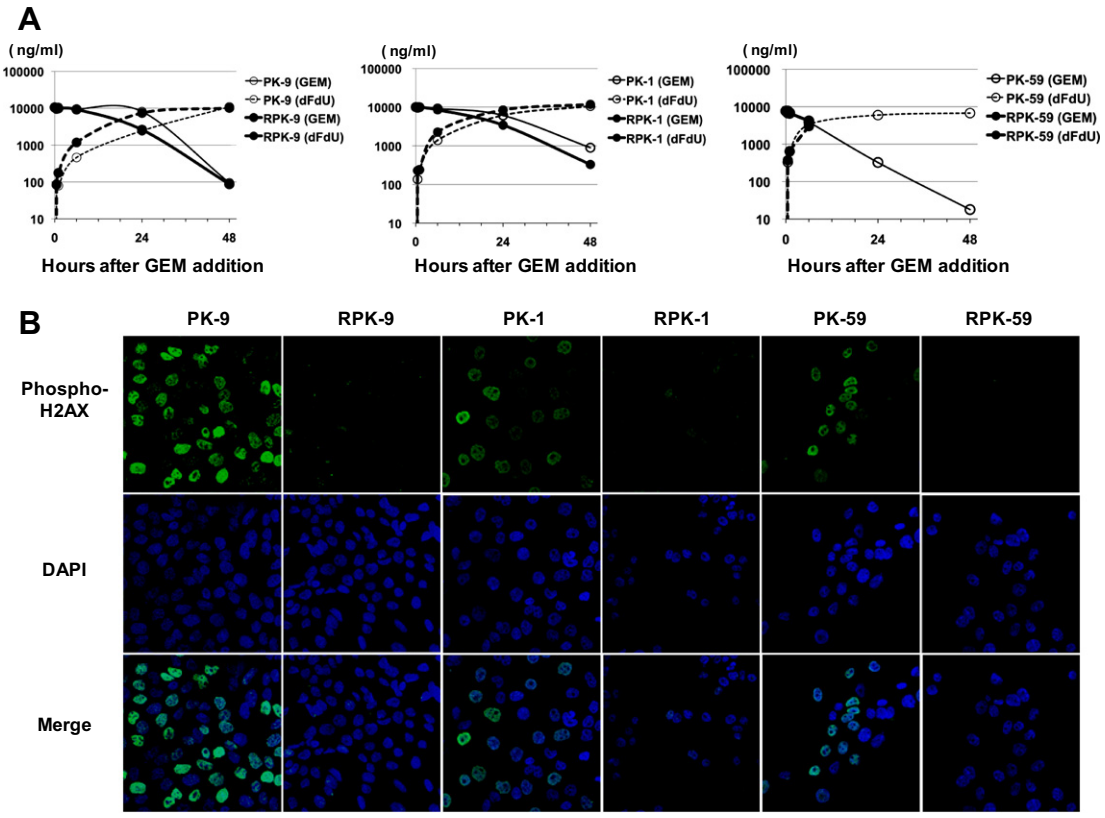

Fig. 2. (A) Time-dependent changes in the concentration of GEM and dFdU in the culture medium after an addition of 10 μg/ml of GEM. Results of three sets of parental and derived resistant cell lines (PK-9 and RPK-9, PK-1 and RPK-1, and PK-59 and RPK-59) are shown. (B) Detection of phospho-H2AX by confocal microscopy 6 h after exposure to 10 μg/ml of GEM. These experiments were performed twice, and representative images are shown.

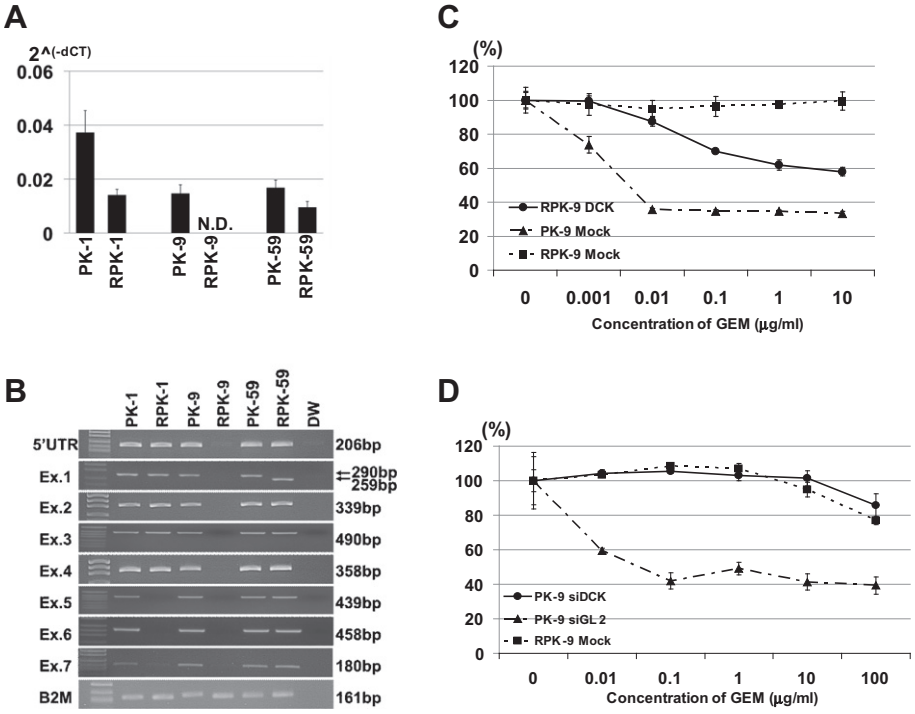

Fig. 3. Inactivation of *DCK* in acquired GEM-resistant cell lines. (A) Levels of *DCK* mRNA in the parental and GEM-resistant cells as determined by qRT-PCR. Data are presented as the mean ± S.D. from three independent experiments. (B) PCR assessment of all the seven exons of the *DCK* gene. (C) GEM sensitivity of RPK-9 after introduction of *DCK* expression vector, pcDNA-DCK. The cell viability was determined 60 h after GEM addition. Mock indicates transfection of an empty vector (pcDNA6/*myc*-HisA) as a negative control. These experiments were performed in quadruplicate, and data are presented as the mean ± S.D. (D) GEM sensitivity after abrogation of *DCK*. PK-9 was transfected with siRNA against *DCK*, and the cell viability was determined 60 h after addition of GEM. Si*GL2* is served as an experimental control. These experiments were performed in quadruplicate, and data are presented as the mean ± S.D.

Table 1
Average expression levels and fold changes of the GEM metabolism-related genes.

Gene	Probe	PK-1*	RPK-1*	Fold change	PK-9*	RPK-9*	Fold change	PK-59*	RPK-59*	Fold change
SLC28A1	A_23_P14667	0.7	6.1	8.2	5.3	1.8	0.3	0.7	32.1	47.7
SLC28A2	A_23_P48816	0.7	3.8	5.3	−2.0	0.9	N/A	−2.6	2.8	N/A
SLC28A3	A_23_P32078	−9.6	4.6	N/A	2.1	4.1	2.0	7.0	7.6	1.1
SLC29A1	A_23_P133694	7579.9	6265.0	0.8	3605.2	2406.3	0.7	3018.6	2779.6	0.9
SLC29A2	A_23_P98294	160.1	159.5	1.0	122.6	295.0	2.4	87.4	62.2	0.7
SLC29A2	A_23_P104705	377.3	334.7	0.9	320.5	1329.7	4.1	173.3	100.7	0.6
CDA	A_23_P34597	110.0	426.7	3.9	204.2	55.0	0.3	2083.0	2308.0	1.1
DCK	A_23_P259438	995.1	3.5	3.5×10^{-3}	293.8	−4.8	N/A	487.7	341.8	0.7
DCK	A_24_P89080	284.4	1.2	4.1×10^{-3}	127.1	−3.7	N/A	215.9	154.9	0.7

* Average data are shown; N/A, not applicable.

we further established single-cell derived subclones by the penicillin cup method; differences in *in vitro* growth patterns were observed in these established subclones.

2.2. Cell proliferation assay

Cells were seeded (5×10^3 cells for PK-1, RPK-1, PK-9 and RPK-9, and 2×10^4 cells for PK-59 and RPK-59) in each well of flat-bottomed 24-well plates in quadruplicate and cultured in 500 μl of medium with concentrations of GEM ranging from 1 ng/ml to 10 μg/ml. Cultures were then allowed to proceed for the indicated time. At that stage, the medium was replaced with 500 μl of 5% alamarBlue® (AbD SEROTEC, Oxford, UK), and, after 3 h incubation, absorption was measured at 590 nm. At least three independent proliferation assays were performed in each experiment.

2.3. Mutational analyses of the KRAS, TP53, CDKN2A, SMAD4, EGFR and DCK

Genetic alterations were analyzed as described previously [13]. Briefly, the DNAs were purified by the phenol/chloroform method, and mutations of *KRAS, TP53, CDKN2A, SMAD4, EGFR* and *DCK* were characterized by PCR-amplification and direct sequencing using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and ABI PRISM 310 DNA sequencer (Applied Biosystems). Nucleotide sequences of primers are listed in Supplementary Table 1 [14–19].

2.4. Concentration measurement of gemcitabine and 2′,2′-difluorodeoxyuridine (dFdU) in the culture medium by LC/MS/MS analysis

LC/ESI-MS/MS was performed using an API 5000 mass spectrometer (Applied Biosystems, Framingham, MA) coupled with Nanospace SI-2 LC system (Shiseido, Kyoto, Japan). The concentrations of GEM and its inactive metabolite 2′,2′-difluorodeoxyuridine (dFdU) were determined by methods previously reported [20] with some modifications (Shimada et al. MS in preparation). After addition of GEM to the cultured cells, the medium was collected at the indicated time. The medium was separated from the cell debris by centrifugation at 1500 g for 10 min, and the aliquots were stored at −80 °C until analysis.

2.5. Immunohistochemistry

The cells were cultured in 8-well culture slides (BD Biosciences, Bedford, MA) with (10 μg/ml) or without GEM for 6 h. Drug-treated cultures were fixed in 4% paraformaldehyde for 15 min, and permeabilized with 0.5% TritonX-100 for 20 min. After blocking with PBS containing 5% goat serum for 30 min, cells were incubated with an anti-phospho-histone H2A.X (Ser139) monoclonal antibody (Millipore, Billerica, MA) at a 400-fold dilution in 3% goat serum in PBS for 2 h, followed by three washes with PBS. Cells were incubated for 1 h with FITC-conjugated goat anti-mouse IgG secondary antibody (Zymed Laboratories Inc., South San Franscisco, CA), followed by three washes with PBS. Cell nuclei were stained with DAPI. Fluorescence was viewed with a Zeiss LSM5 PASCAL confocal microscope system (Carl Zeiss Inc., Thornwood, NY) at the Biomedical Research Core of Tohoku University School of Medicine.

2.6. Microarray analysis

Microarray analyses were performed according to previously described methods [21]. In brief, total RNAs were isolated from the pancreatic cancer cell lines with RNeasy Mini Kit (Qiagen, Studio City, CA) and labeled with Cy-3 using Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA). Each aliquot of 1.65 μg of cRNA was assembled and hybridized to Agilent whole human genome microarray (4 x 44 K); slides were scanned with the Agilent G2565BA microarray scanner (Agilent Technologies). Intensity data from microarray images were extracted with Feature Extraction Software 9.5.1 (Agilent Technologies). These experiments were performed in duplicate, and results were analyzed using the GeneSpring software (Silicone Genetics, Redwood City, CA).

2.7. Quantitative reverse transcription PCR (qRT-PCR)

Each aliquot of 2 μg total RNA was reverse transcribed to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. The mRNA expression level was determined by the methods described previously [22] using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Expression of β-2-microglobulin (*B2M*) was used as the internal control. The nucleotide sequences for primers and probes are listed in Supplementary Table 1. Amplifications were carried out in the 15 μl reaction mixtures in triplicate using Absolute QPCR ROX Mix (Thermo Fisher Scientific, Leicestershire, UK) according to the manufacturer's recommended method. The expression of target genes relative to *B2M* expression was calculated as follows: 2^(dCT) (dCt = Ct [target] - Ct[B2M]). Independent triplicate assays were performed.

2.8. Introduction of DCK into acquired resistant cells

The open reading frame of *DCK* was PCR-amplified by KOD polymerase (Toyobo, Osaka, Japan) using a human fetal brain cDNA library (Clontech, Pala Alto, CA) and a pair of primers (Supplementary Table 1) equipped with the Kozak consensus sequence and a restriction recognition site of either *Bam*HI or *Xho*I to facilitate directional cloning in expression vector pcDNA6/*myc*-HisA (Invitrogen, Carlsbad, CA). The full length *DCK* open reading

frame excluding the termination codon, termed pcDNA-DCK, was then cloned. Each aliquot of 5×10^5 cells of GEM-resistant cell line was cultured in 6 cm dishes for 15 h and transfected with 4 μg of pcDNA-DCK using the Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. After 24 h, cells were collected, and 2×10^4 cells were transferred to each well of flat-bottomed 24-well plates to determine their viability with (10 μg/ml) or without GEM using alamarBlue® (AbD SEROTEC).

2.9. Knockdown of DCK in parental cells

Each aliquot of 5×10^5 cells of a parental cell line was cultured in 6 cm dishes for 15 h and transfected with siRNA against *DCK* at the appropriate concentration using Lipofectamine™ 2000 reagent. After 48 h, an aliquot of 2×10^4 cells was transferred to each well of flat-bottomed 24-well plates for the proliferation assay. siRNA against the luciferase gene (*GL2*) [23] was used as a negative control. Nucleotide sequences of siRNAs were listed in Supplementary Table 1. These siRNAs were purchased from Integrated DNA Technologies (Coralville, IA). The efficiency of *DCK* knockdown was confirmed by qRT-PCR (data not shown).

2.10. Statistical analyses

All experiments were performed in duplicate or triplicate. A two-tailed Student's *t*-test was used for statistical analyses of comparative data using Microsoft Excel software (Microsoft Corporation, Tokyo, Japan). Values of $P < 0.05$ were considered as significant.

3. Results and discussion

3.1. Establishment and characterization of acquired GEM-resistant cancer cells

The GEM resistant three pancreatic cancer cell lines were first established by methods described in Materials and methods. Three other cell lines were also established. Morphological features of the established resistant cell lines as well as those of the parental cell lines are shown in Fig. 1A, and sensitivities to GEM are shown in Fig. 1B; the parental cells were variably sensitive to GEM, whereas proliferations of GEM-resistant pancreatic cancer cell lines were not suppressed by increasing doses of GEM. These results demonstrate that GEM-resistance is sustainable. We also analyzed the expression and mutation of five genes, *KRAS, TP53, CDKN2A, SMAD4, and EGFR,* and found that there were no acquisitions of the genomic alteration in the established resistant cell lines (Supplementary Table 2); chemoresistance-acquisition against GEM does not depend on mutation of these genes.

Because PK-9, PK-1 and PK-59 were high-, moderately-, and low- GEM sensitivity cell lines, respectively [24], we established single-cell derived subclones using their derived resistant cell lines (RPK-9, RPK-1 and RPK-59). Around 30 subclones were established from each resistant cell line, and we divided them into three groups (island type, sheet type, and degenerative type) based on their *in vitro* growth patterns (Supplementary Fig. 1). These distinct growth patterns were maintained during passages.

3.2. Preserved influx and efflux of gemcitabine in GEM-resistant cell lines

Decreased influx and increased efflux of GEM have been suggested to be involved in GEM resistance [25]. To assess the influx and efflux of GEM in our cell lines, we serially measured the concentration of GEM and its inactive metabolite, 2′,2′-difluorodeoxyuridine (dFdU), in the culture medium after an addition of GEM (10 μg/ml) using the LC/ESI-MS/MS method (Fig. 2A). The decline in the concentration of GEM and increase of dFdU in the culture medium were essentially equal between the parental and corresponding GEM-resistant cell lines. These results suggested that the alterations of influx and/or efflux of GEM are not major contributers to acquisition of GEM-resistance in our cell lines.

3.3. Diminished GEM-induced H2AX phosphorylation in GEM-resistant cell lines

After GEM enters the cells, GEM is phosphorylated to a triphosphate form and incorporated into DNA [26]. Incorporation induces steric hindrance of extending replication forks, leading to fork stalling, as detected by H2AX phosphorylation [27]. To determine whether there are any differences in GEM-induced stalled replications between the parental and GEM-resistant cells, we performed an immunofluorescent detection of phosphorylation of H2AX. As shown in Fig. 2B, distinct γ-H2AX foci were evident in the nuclei of all the parental cells, whereas γ-H2AX foci were very rarely seen in the nuclei of all the GEM-resistant cells. These results suggest that GEM is not incorporated into the DNA of the GEM-resistant cells.

3.4. Expression of GEM metabolism-related genes

Using three sets of parental and resistant cell lines (PK-9 and RPK-9, PK-1 and RPK-1, and PK-59 and RPK-59), we studied mRNA expression levels by microarray analyses; data are available in the Gene Expression Omnibus database (http://www.ncbi.nlom.nih.gov/geo) under the accession number GSE35141. *ADAM28* and *TLE4* were identified as commonly upregulated genes in all the three paired sets of parental and resistant cell lines. However, the expression levels of these genes in single-cell derived subclones showed inconsistency among the clones, and siRNA-mediated knockdown of these genes did not alter GEM sensitivities, although specific knockdown was observed (data not shown).

GEM enters the cell *via* nucleoside transporters. These include solute carrier family 28 (sodium-coupled nucleoside transporter) members such as SLC28A1, SLC28A2, SLC28A3, and/or solute carrier family 29 (nucleoside transporter) members such as SLC29A1 and SLC29A2. After intracellular uptake, GEM is phosphorylated by *DCK* and subsequently converted to the active form of GEM. Cytidine deaminase (CDA) inactivates GEM by deamination [25]. To identify the key molecules associated with GEM resistance, we re-analyzed microarray data between parental and GEM-resistant cell lines. GEM metabolism-related genes were specifically examined (see Table 1), *SLC28A1*, *SLC28A2* and *SLC28A3* were rarely expressed in any of the cell lines. *SLC29A1* and *SLC29A2* were expressed in all the three parental cell lines and were preserved in the GEM-resistant cells; these results suggest that GEM is transported equally into parental and GEM-resistant cell lines. These results are compatible with those from the medium concentration study of GEM and dFdU (Fig. 2). Compared with its parental cells, the expression of *CDA* was augmented only in the RPK-1 cells but not in RPK-9 or RPK-59. However, dFdU, a metabolite of GEM, was not increased in RPK-1 by LC/ESI-MS/MS analyses. Thus, a deamination process by *CDA* is not likely to be a mainstream event in GEM resistance acquisition. Strikingly, *DCK* was almost obliterated in the RPK-1 and RPK-9 cells. The RPK-59 cells also had reduced *DCK* expression; these results were confirmed by qRT-PCR (Fig. 3A). Therefore, *DCK* drew our attention as a molecule responsible for GEM resistance.

3.5. Genetic alterations of DCK

Although genetic alterations of *DCK* in GEM-resistant pancreatic cancer have not been documented, inactivating mutations of *DCK* in human cancer cell lines have been reported in melanoma, leukemia, lymphoma, fibrosarcoma, and ovarian cancer cell lines, as well as in leukemic blasts from AML patients who were resistant to pyrimidine nucleoside analogs [15,28–30]. Therefore, we performed mutation analyses of *DCK*. As shown in Fig. 3B, striking alterations were only observed in resistant cell lines; either total or partial homozygous deletions in RPK-9 and RPK-1, and a 31-bp homozygous deletion in RPK-59 that would cause a frameshift followed by truncation of the protein product were identified (Supplementary Fig. 2). The other three resistant cell lines (RDLD-1, RMKN-28 and RTFK-1) were also characterized by mutations in *DCK*, and the mutation was observed only in resistant cell lines; W92X in RDLD-1 and a homozygous deletion of exons 1 and 2 in RTFK-1 (Supplementary Fig. 2). These results are summarized in Supplementary Table 2; inactivation of *DCK* seems to play an important role in acquired resistance against GEM in our series of cancer cell lines.

3.6. Overexpression and knockdown of DCK

To determine the importance of *DCK* deletion in the acquisition of the GEM-resistance, we introduced *DCK* into resistant cell lines and siRNAs into parental cell lines for knockdown. Representative results using RPK-9 and PK-9 are shown in Figs. 3C and D; restoration of *DCK* in the GEM-resistant cell line resumed GEM sensitivity, and siRNA-mediated knockdown of *DCK* in the parental cell line resulted in acquisition of GEM resistance. These results clearly indicate that the inactivation of *DCK* plays a crucial role of acquiring GEM-resistance.

3.7. Future studies and conclusion

In this study, we have revealed a central role of *DCK* inactivation in the acquired resistance to GEM. Inactivating mutations of *DCK* are likely to be observed in clinically resected tissues subjected to GEM therapy. In an attempt to elucidate the clinical relevance of this discovery, we are currently collecting clinical tissue samples from relapsed cancer in patients who have been subjected to GEM administration.

Recently, the degree of *DCK* protein expression in resected pancreatic adenocarcinomas has been identified as an independent and strong prognostic factor for patients with adjuvant gemcitabine therapy [31]. Hence, monitoring *DCK* expression may also be a good indicator for response to GEM therapy. Once *DCK* mutations are identified, other therapeutic strategies to overcome *DCK* deficiency are necessary. S-1, an oral fluoropyrimidine containing tegafur, has been reported to be effective for metastatic pancreatic cancer [32]. Because tegafur in S-1 is metabolized to 5-FU mainly in the liver, we also analyzed the effectiveness of this drug; our preliminary results show that 5-FU killed both the parental and GEM-resistant pancreatic cancer cells equally (Manabe et al. MS in preparation). These results support the rationale for combination therapy using GEM and S-1, and we recommend S-1 as a good choice against *DCK* deficient pancreatic cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.03.122.

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