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Acquisition of chemoresistance to gemcitabine is induced by a loss-of-function missense mutation of *DCK*

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ABSTRACT

The anti-tumor activity of gemcitabine (GEM) has been clinically proven in several solid tumors, including pancreatic cancer, biliary tract cancer, urinary bladder cancer, and non-small cell lung cancer. However, problems remain with issues such as acquisition of chemoresistance against GEM. GEM is activated after phosphorylation by deoxycytidine kinase (DCK) inside of the cell; thus, DCK inactivation is one of the important mechanisms for acquisition of GEM resistance. We previously investigated the *DCK* gene in multiple GEM resistant cancer cell lines and identified frequent inactivating mutations. In this study, we identified two crucial genetic alteration in *DCK*. (1) A total deletion of *DCK* in RTGBC1-TKB, an acquired GEM resistant cell line derived from a gall bladder cancer cell line TGBC1-TKB. (2) An E197K missense alteration of *DCK* in MKN28, a gastric cancer cell line; its acquired GEM resistant cancer cell line, RMKN28, showed a loss of the normal E197 allele. We introduced either normal DCK or altered DCK_E197K into RMKN28 and proved that only the introduction of normal DCK restored GEM sensitivity. Furthermore, we analyzed 104 healthy volunteers and found that none of them carried the same base substitution observed in MKN28. These results strongly suggest that (1) the E197K alteration in *DCK* causes inactivation of DCK, and that (2) loss of the normal E197 allele is the crucial mechanism in acquisition of GEM resistance in RMKN28.

1. Introduction

Gemcitabine (2′,2′-difluoro 2′-deoxycytidine, dFdC), abbreviated GEM, is a deoxycytidine analogue with antitumor activity that is widely used for treatment of several solid tumors, including pancreatic cancer, biliary tract cancer, urinary bladder cancer, and non-small cell lung cancer. Acquisition of resistance to GEM by tumor cells is one of the major causes of treatment failure during the course of chemotherapy. Several studies on the mechanisms of resistance to GEM have identified some candidate genes that are associated with such resistance [1].

In our previous study, we established multiple GEM resistant cancer cell lines and identified a frequent inactivating mutation of deoxycytidine kinase (*DCK*) in these lines [2]. DCK is the enzyme involved in the first rate-limiting phosphorylation cascade. Gemcitabine triphosphate (dFd-CTP) is incorporated into DNA, where it inhibits DNA synthesis [3]; thus inactivation of DCK function blocks activity of GEM [2].

In this study, we have investigated a GEM resistant gall bladder cancer cell line and a gastric cancer cell line and identified an inactivating mutation of DCK, including a total deletion in the former and a novel single nucleotide substitution in the latter causing a loss of DCK function.

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2. Materials and methods

2.1. Establishment of GEM-resistant cell lines

TGBC1-TKB, a gall bladder cancer cell line, was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT (Tsukuba, Japan). MKN28 was used in our previous study [2]. Gemcitabine hydrochloride (GEM) was purchased from Eli Lilly (Indianapolis, IN). In brief, each individual cell line at the subconfluent density was exposed to GEM at the concentration for its respective IC_{50} for more than one month. When surviving cell colonies were observed, the GEM concentration was gradually increased in a stepwise pattern to a final concentration of 10 μg/ml. Each candidate GEM-resistant cell line was grown without GEM for more than one month and then exposed to GEM again at 10 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 (Resistant) as the prefix. RMKN28 was established in our previous study [2], and RTGBC1-TKB was established in this study.

2.2. Cell proliferation assay

Cells were seeded at 5×10^3 cells in each well of flat-bottomed 24-well plates in quadruplicate and cultured in 500 μl of medium with sequential one order in magnitude different concentrations of GEM (from 0.001 μg/ml to 10 μg/ml). Cultures were then allowed to proceed for indicated times. At the end point, the medium was replaced with 500 μl of 5% alamarBlue (AbD SEROTEC, Oxford, UK), and, after 3 h of incubation, absorbance was measured at 590 nm. At least two independent proliferation assays were performed.

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

Genetic alterations were analyzed as described previously [2]. Briefly, DNAs were purified using the DNeasy Blood & Tissue Kit (Qiagen, Studio City, CA), and mutations of the *KRAS, TP53, CDKN2A, SMAD4,* and *DCK* genes were characterized by direct sequencing of the PCR amplified products using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI PRISM 310 DNA Sequencer (Applied Biosystems). Nucleotide sequences of primers used and their conditions are listed in Supplementary Table S1.

2.4. 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 using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Expression of β-2 microglobulin (*B2M*) was used as the internal control. Amplifications were carried out in 15 μl reaction mixtures in triplicate using Absolute QPCR Mixes (Thermo Scientific, Waltham, MA) according to the manufacturer's recommendations. Expression levels of target genes relative to *B2M* were calculated as follows: 2^dCT (dCt = Ct [target] - [B2M]). Independent triplicate assays were performed. Primer sequences and PCR conditions were listed in Supplementary Table S1.

2.5. Establishment of DCK-wild type or DCK-E197K stably expressing RMKN28 cells

The expression vector of wild type DCK, pcDNA-DCK, was constructed as described previously [2]. The DCK_E197K expression vector was constructed using RT-PCR amplified cDNA from RMKN28 and the pcDNA6/myc-HisA vector (Invitrogen, Carlsbad, CA) by the methods previously described [2]. Transfection into cells was performed using Lipofectamine 2000 reagent (Invitrogen). In order to establish stable cell line (DCK in RMKN28 or DCK_E197K in RMKN28), transfected cells were continuously treated with 5 μg/ml of Blasticidin S (Funakoshi, Tokyo, Japan).

2.6. Immunohistochemistry

Cells were cultured in 8-well culture slides (BD Bioscience, Bedford, MA) with 10 μg/ml GEM or without GEM for 6 h. Cultured cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.05% Triton-100 for 20 min. After blocking with PBS containing 5% goat serum for 30min, cells were incubated with anti-phospho-histone H2AX (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. The cells were incubated with FITC-conjugated goat anti-mouse IgG secondary antibody (Zymed Laboratories Inc., South San Francisco, CA) for 1 h. After three washes with PBS, cell nuclei were stained with DAPI. Fluorescence was observed with a Zeiss LSM5 PASCAL confocal microscope system (Carl Zeiss Inc., Thornwood, NY) at the Biomedical Research Core of Tohoku University School of Medicine.

2.7. Microarray analysis

Microarray analyses were performed according to methods described previously [4]. In brief, total RNAs isolated using RNeasy Mini Kit (Qiagen, Studio City, CA) were labeled with Cy-3 using Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA), and each aliquot of 1.65 μg of cRNA was assembled and hybridized to an Agilent whole human genome microarray (4 × 44K). Microarray 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) at the Biomedical Research Core of Tohoku University School of Medicine. The experiments were performed in duplicate. Obtained results were analyzed using the GeneSpring software (Silicone Genetics, Redwood City, CA).

2.8. Capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) analysis for quantification of GEM and its metabolites

A total of 1.0×10^5 cells were seeded in a 6-well plate and allowed to adhere for 12 h. After adding 2 μg/ml GEM, the incubated medium and cell components were collected at the indicated time. To gain cell components, the cells were washed twice with excess amount of 5% mannitol and then 600 μl of MeOH containing 25 μM each of methionine sulfone (# A17027) (Alfa Aesar, Ward Hill, MA) and camphor-10-sulfonic acid (Wako 037-01032) (Wako, Osaka, Japan) were added as internal standards. After leaving the mixture at rest for 10 min, 400 μl of the sample solution was transferred into a new tube; then 400 μl of $CHCl_3$ and 160 μl of Milli-Q water were added and mixed well. The mixture was centrifuged at 10,000 g for 3 min at 4 °C, and the upper aqueous layer (450 μl) was filtered through a centrifugal filter (5-kDa cut-off filter) (Millipore, Billerica, MA) at 9,100 g for 3 h at 4 °C. The filtrate was concentrated centrifugally and dissolved in 25 μl of Milli-Q water containing 200 μM each of 3-aminopyrrolidine (# 404624) (Sigma–Aldrich, St. Louis, MO) and trimesic acid (# 206-03641) (Wako, Osaka, Japan) as reference compounds prior to CE-TOFMS analysis.

Metabolomic profiling of GEM and its metabolites were performed as described previously [5]. Metabolite identifications were