

# Silencing of *ECHDC1* inhibits growth of gemcitabine-resistant bladder cancer cells

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**Abstract.** Combined gemcitabine and cisplatin (GC) treatment is a first line chemotherapy for bladder cancer. However, acquired resistance to GC has been a major problem. To address the mechanism of gemcitabine resistance, and to identify potential biomarkers or target proteins for its therapy, we aimed to identify candidate proteins associated with gemcitabine resistance using proteomic analysis. We established gemcitabine-resistant human bladder cancer cell lines (UMUC3GR and HT1376GR) from gemcitabine-sensitive human bladder cancer cell lines (UMUC3 and HT1376). We compared the protein expression of parental and gemcitabine-resistant cell lines using isobaric tags for relative and absolute quantification (iTRAQ) and liquid chromatography tandem mass spectrometry. Among the identified proteins, ethylmalonyl-CoA decarboxylase (ECHDC1) expression was significantly increased in both of the gemcitabine-resistant cell lines compared to the respective parental cell lines. Silencing of *ECHDC1* reduced ECHDC1 expression and significantly inhibited the proliferation of UMUC3GR cells. Furthermore, silencing of *ECHDC1* induced upregulation of p27, which is critical for cell cycle arrest in the G1 phase, and induced G1 arrest. In conclusion, ECHDC1 expression is increased in gemcitabine-resistant bladder cancer cells, and is involved in their cell growth. ECHDC1, which is a metabolite proofreading enzyme, may be a novel potential target for gemcitabine-resistant bladder cancer therapy.

## Introduction

Worldwide, there were 429,800 new cases of and 165,100 deaths due to bladder cancer in 2012 (1). Gemcitabine

(2',2'-difluoro-2'-deoxycytidine) is an important drug for treating cancers including bladder cancer. The combination of gemcitabine and cisplatin (GC) has been standard chemotherapy for metastatic bladder cancer and for muscle invasive bladder cancer as a neoadjuvant chemotherapy. GC is effective for about half of patients with advanced or metastatic bladder cancer. In a phase III study (2), the response rate was 49%. In our hospital, the response rate for GC was reported as 44% (3). However, the many of these patients later developed progressive disease.

Biomarkers associated with and molecular mechanism of gemcitabine resistance acquisition in bladder cancer are not fully understood. It is possible that a protein that is more highly expressed in gemcitabine-resistant bladder cancer may be a gemcitabine-resistant biomarker or therapeutic target. Proteomic analysis is an ideal method for identification of such a protein and indeed, in recent years, proteins associated with chemoresistance have been successfully identified using proteomic analysis (4-6). Here, we used the method of isobaric tags for relative and absolute quantification (iTRAQ method), which can compare the protein levels of more than three samples in proteomic analysis (7).

In the present study, to identify a protein associated with gemcitabine resistance, we established gemcitabine-resistant human bladder cancer cell lines (UMUC3GR, HT1376GR) that we derived from human bladder cancer cell lines (UMUC3 and HT1376). We analyzed these cell lines at the protein level using the iTRAQ method, liquid chromatography, and tandem mass spectrometry (MS/MS). In addition, we further analyzed a protein that was found to be more highly expressed in gemcitabine-resistant cell lines, using biochemical and molecular biological techniques.

## Materials and methods

**Cell culture.** The human bladder cancer cell lines, UMUC3 and HT1376, which were used in this study, were purchased from DS Pharma Biomedical (Osaka, Japan). UMUC3 cells were maintained in minimum essential medium (MEM) supplemented with MEM non-essential amino acids (NEAA) and sodium pyruvate (Gibco, St. Louis, MO, USA). HT1376 cells were maintained in RPMI-1640 medium (Wako, Osaka, Japan). Both media were supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated

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in a humidified incubator at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air. Each gemcitabine-resistant cell line (GR) was obtained from the parental UMUC3 or HT1376 cells.

The UMUC3 and HT1376 cells were grown in cell culture media containing gemcitabine (Wako), starting with a concentration of 10–2 µM. The cells were then passaged through stepwise increasing concentrations of gemcitabine up to a concentration of 50 µM. The cells were repeatedly passaged at each gemcitabine concentration in the stepwise gradient.

iTRAQ proteomic analysis with Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. The four human bladder cancer cell lines HT1376, HT1376GR, UMUC3, and UMUC3GR were each grown to 80% confluency, following which the cellular proteins were extracted using Mammalian Protein Extraction Reagents (M-PER; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, Inc.). The protein concentration of the cellular lysate was adjusted to a concentration of 1 µg/µl using dissolution buffer. Each sample was digested with 1 µg/µl trypsin solution (AB SCIEX, Framingham, MA, USA) at 37°C for 24 h and was then desalted. Peptide samples from each of the cell lines were labelled using the iTRAQ® Reagent-multiple Assay kit (AB SCIEX) as follows: HT1376 with the 116 tag, HT1376GR with the 117 tag, UMUC3 with the 118 tag, and UMUC3GR with the 119 tag. All samples were mixed and fractionated using strong cation exchange chromatography (SCX) with a Cation Exchange Buffer Pack (AB SCIEX). The peptide sample from each SCX fraction was enriched using a trap column (HiQ sil C18; KYA Technologies, Tokyo, Japan) and was then separated on an electrospray ionization (ESI) column (HiQ sil C18P-3; KYA Technologies, Tokyo, Japan) at a flow rate of 150 nl/min. MS/MS analysis of peptide samples was carried out using the Triple TOF™ 5600 system (AB SCIEX) interfaced with the DiNa system (LC) (KYA Technologies, Tokyo, Japan).

MS and MS/MS data searches were carried out using ProteinPilot™ software 4.5 (AB SCIEX). Searches used the UniProtKB (<http://www.uniprot.org>) database. The false discovery rate (FDR) was calculated and high-confidence protein identifications were obtained by using a Global FDR from Fit 1.0% at the peptide level. Quantitative estimates provided for each protein by ProteinPilot were utilized: the fold change ratios of differential expression between labeled protein extracts, and the P-value representing the probability that the observed ratio is different than 1 by chance. We selected 1.5-fold-change as a cutoff to classify upregulated proteins.

**Western blot analysis.** Cells were lysed with cell lysis buffer containing phenylmethanesulfonyl fluoride (Cell Signaling Technology, Inc., Danvers, MA, USA) and protease inhibitor cocktails (Sigma-Aldrich). Samples were centrifuged at 14,000 × g for 10 min at 4°C, and supernatants were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% skimmed milk, the membranes were probed with primary antibodies against β-actin, p27 (Cell Signaling Technology, Inc.) and ethylmalonyl-CoA decarboxylase (*ECHDC1*, Abcam) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibody (Cell

Signaling Technology, Inc.) for 1 h at room temperature. The immune complexes were visualized with the Enhanced Chemiluminescence Plus detection system (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. The signal was quantified using ImageJ and normalized to that of β-actin.

**Immunofluorescence.** Cells were seeded in an 8-well chamber slide (Thermo Fisher Scientific, Inc.) and incubated for 24 h. The cells were fixed with 4% paraformaldehyde followed by blocking with 1% bovine serum albumin. The cells were incubated with an anti-*ECHDC1* antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h. Thereafter, they were incubated with fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min. Hoechst 33342 (Invitrogen Life Technologies, Carlsbad, CA, USA) was used for nuclear staining. Fluorescence was photographed using a BZ9000 Fluorescence microscope (Keyence Corporation, Osaka, Japan).

**Transfection.** Silencer Select Negative control #1 siRNA (Life Technologies Corp., Carlsbad, CA, USA) or Silencer Select *ECHDC1*: Ethylmalonyl-CoA decarboxylase1 siRNA (s229273; Life Technologies Corp.) was added to the adherent cells at a final concentration of 5 nM using Lipofectamine® RNAiMAX (Invitrogen Life Technologies) as the transfection reagent for 24 h.

**Drug cytotoxicity analysis and real time analysis of cell proliferation.** Cells were seeded in 96-well plates at a density of 3×10<sup>3</sup> cells/well and were cultured with graded concentrations of gemcitabine in at least three replicate wells at 37°C. At 72 h after gemcitabine exposure, the relative effect of gemcitabine on the proliferation of each cell line was assessed by using the Cell Counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Absorbance at 450 nm was determined using a spectrophotometer (Thermo Scientific Multiskan FC; Thermo Fisher Scientific, Inc.). The absorbance of cells not treated with gemcitabine was considered to be 100%.

Real-time analysis of cell proliferation was performed using impedance measurement with the xCELLigence system. Cell proliferation (Cell Index) was checked using the xCELLigence Real-Time Cell Analyzer (RTCA) instrument according to the instructions of the supplier (Roche Applied Science and ACEA Biosciences, San Diego, CA, USA). This system has been extensively used in other studies (8,9). The xCELLigence system can quantify the electrical impedance across electrodes at the bottom of each well of the tissue culture plates. Impedance changes reflect cell numbers, and cell viability is expressed as Cell Index values. Cells were seeded at a density of 8×10<sup>3</sup> cells/well in a specialized 8-well plate (E-plate) used with the RTCA xCELLigence instrument and the experiment was allowed to run for 96 h at 37°C. Cell Index values were recorded at 15 min interval sweeps until the end of the experiment. We normalized the Cell Index at 24 h after seeding the cells.

**Cell cycle analysis.** Cells were collected using Accutase (Innovative Cell Technologies, San Diego, CA, USA). Cell

cycle analysis was performed using the CycleTest Plus DNA reagent kit (BD Biosciences, San Jose, CA, USA). The data were analyzed using ModFitLT (Verity software House, Topsham, ME, USA) to generate percentages of cells in G0/G1, S and G2/M phases. At least 18,000 cells were analyzed in each experiment.

**Statistical analysis.** Quantitative data are expressed as means  $\pm$  standard deviation (SD). Statistical significance was assessed using Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Establishment of gemcitabine-resistant bladder cancer cell lines.** The cytotoxicity of gemcitabine towards the four human bladder cancer cell lines HT1376, HT1376GR, UMUC3, and UMUC3GR was examined. Analysis of cell viability using CCK-8 indicated that, while the parental cell lines showed a dose-dependent sensitivity to gemcitabine, the HT1376GR and UMUC3GR cell lines derived from them had acquired gemcitabine resistance (Fig. 1A and B).

**Identification of proteins involved in gemcitabine resistance using iTRAQ proteomic analysis.** A total of 3,930 proteins were identified in the cell lines using iTRAQ proteomic analysis. Of these proteins, the expression of several proteins was increased more than 1.5-fold in the gemcitabine-resistant cells (UMUC3GR and HT1376GR), compared to the corresponding gemcitabine-sensitive parental cells (UMUC3 and HT1376). Only expression of the ECHDC1 protein was significantly increased ( $P < 0.05$ ) in both of the gemcitabine-resistant cell lines (Table I).

**Western blotting and immunofluorescence analysis of ECHDC1 protein expression in gemcitabine-resistant cells.** Western blotting of the four cell lines confirmed a strong increase in ECHDC1 protein levels (34 kDa) in the gemcitabine-resistant cells, UMUC3GR and HT1376GR, compared with its expression in the respective parental UMUC3 and HT1376 cell lines (Fig. 2A). Immunofluorescence analysis also resulted in a much stronger cytoplasmic ECHDC1 protein signal in UMUC3GR cells than in the UMUC parental cells (Fig. 2B).

**Silencing of ECHDC1 significantly inhibited cell proliferation in UMUC3GR cells.** To examine the functional role of the ECHDC1 protein in bladder cancer cells, we knocked down *ECHDC1* in UMUC3GR cells using siRNA. We confirmed using western blotting that ECHDC1 protein expression was significantly reduced in cells transfected with *ECHDC1*-siRNA compared with cells transfected with control siRNA (Fig. 3). We then determined the effect of *ECHDC1* silencing on cell proliferation *in vitro*. RTCA analysis showed that the proliferation of bladder cancer cells was significantly inhibited ( $P < 0.05$ ) by silencing of *ECHDC1* compared to cells transfected with control siRNA (Fig. 4).

**Knockdown of ECHDC1 induced G0/G1 phase cell cycle arrest and upregulation of p27.** To investigate the mechanism of the anti-proliferative effect of knockdown of *ECHDC1* on UMUC3GR cells, we measured the percentage of the

Table I. Proteins with increased expression in gemcitabine-resistant cells identified using iTRAQ proteomic analysis.

Protein name	Gene	Fold-change HT1376GR/HT1376		P-value	Fold-change UMUC3GR/UMUC3		P-value	%Cov(95)	Accession number
Ethylmalonyl-CoA decarboxylase	ECHDC1	1.674	0.034	0.000	3.593	0.000	42.00	42.00	spQ9NTX5
Integrin $\alpha$ -2	ITGA2	3.356	0.359	0.205	1.778	0.205	23.60	23.60	spP17301
Vasodilator-stimulated phosphoprotein	VASP	3.645	0.049	0.131	1.609	0.131	15.50	15.50	spP50552
Isoform 2 of Cleavage stimulation factor subunit 2	CSTF2	1.898	0.264	0.180	1.913	0.180	12.90	12.90	spP33240-2
Scaffold attachment factor B2	SAFB2	1.551	0.039	0.398	1.596	0.398	13.60	13.60	spQ14151
Serine incorporator 1	SERINC1	2.475	0.139	0.286	1.593	0.286	6.60	6.60	spQ9NRX5
Histone deacetylase 3	HDAC3	1.970	0.363	0.084	2.225	0.084	5.40	5.40	spO15379
ATP-dependent RNA helicase SUPV3L1	SUPV3L1	2.134	0.281	0.121	1.705	0.121	3.20	3.20	spQ8IYB8

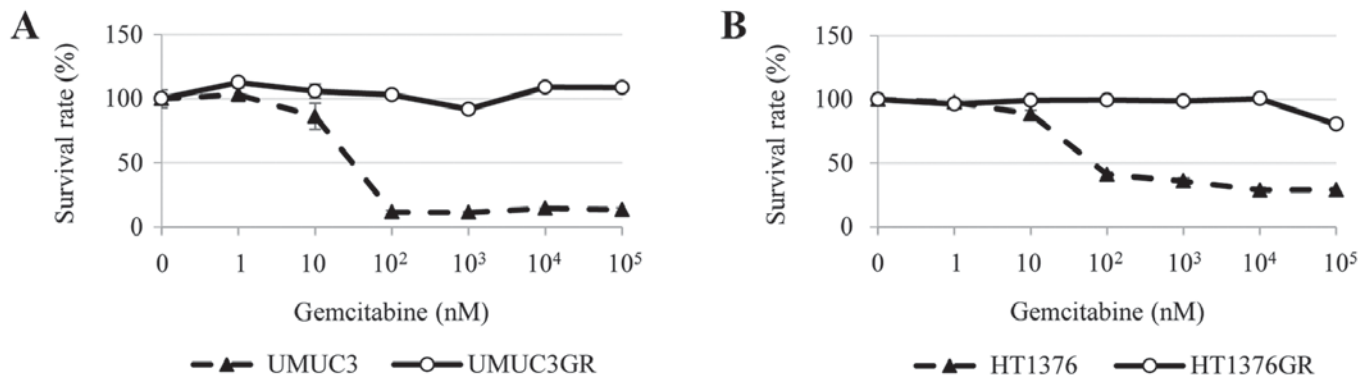


Figure 1. Analysis of gemcitabine resistance of the bladder cancer cell lines. Bladder cancer cells were treated with gemcitabine for 72 h and cell viability was analyzed using CCK-8. The viability of parental UMUC3 (A) and HT1376 (B) cells was respectively suppressed compared to (A) UMUC3GR and (B) HT1376GR by treatment with gemcitabine in a dose-dependent manner. Values are expressed as means  $\pm$  standard deviation.

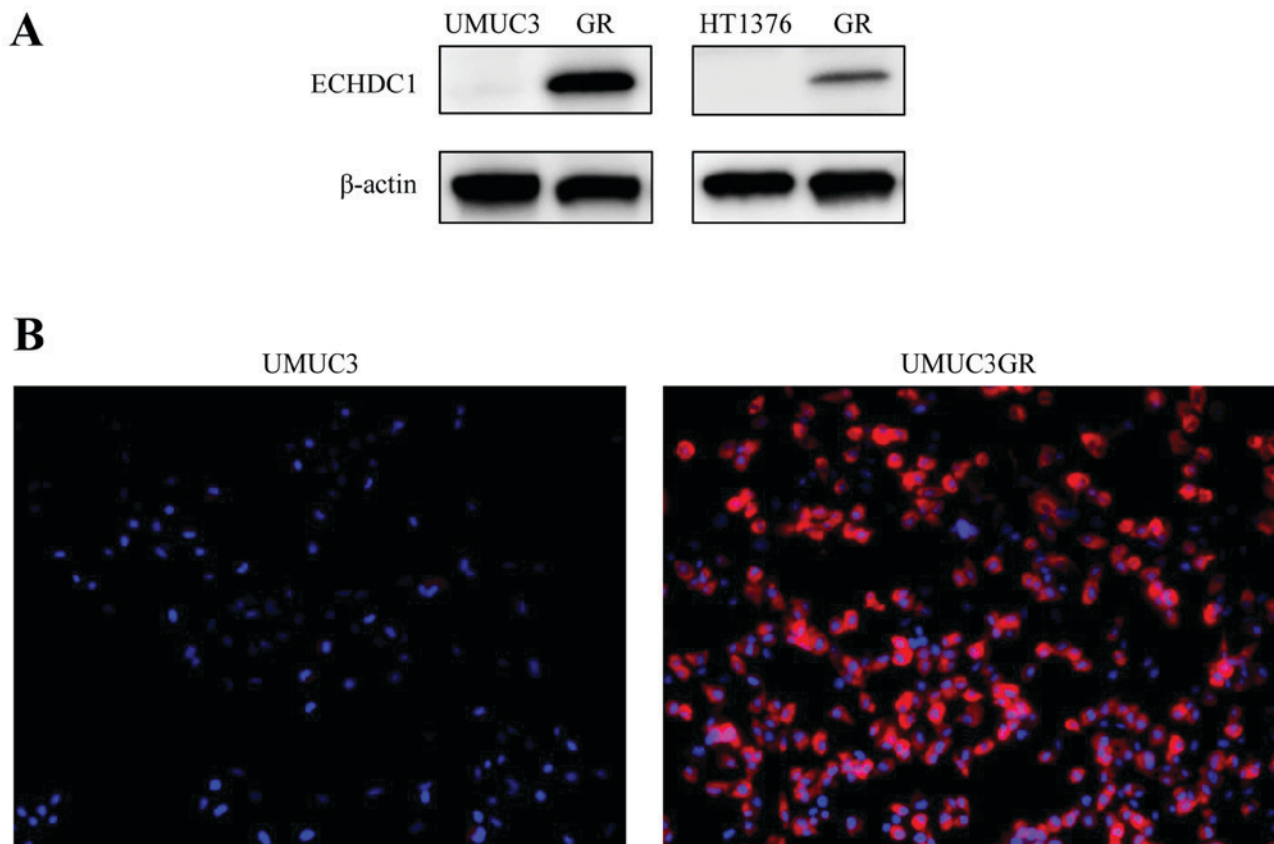


Figure 2. *ECHDC1* protein expression in gemcitabine resistant and sensitive cell lines. (A) *ECHDC1* protein expression levels in parental and gemcitabine resistant (GR) cell lines were determined using western blotting. *ECHDC1* expression levels were clearly increased in the gemcitabine-resistant cells compared to the gemcitabine-sensitive cells. (B) Immunofluorescence analysis of *ECHDC1* expression (red) in the indicated cells. Nuclei were counterstained with Hoechst 33342 (blue). The red signal in the cytoplasm, reflecting *ECHDC1* expression, was much stronger in UMUC3GR than in UMUC3 cells.

cells in different cell cycle phases using flow cytometry with propidium iodide (PI) staining at 48 h after transfection with siRNA against *ECHDC1* or with control siRNA. Knockdown of *ECHDC1* induced significantly higher accumulation of cells in the G0/G1-phase cell compared with the control ( $P < 0.05$ ). Thus, the percentage of G0/G1 phase cells was increased from 43.5% in the control to 60.2% in the knockdown cells, and the percentage of S phase cells was decreased from 38.8 to 27.0% (Fig. 5). To investigate the molecular basis of these changes, we analyzed the expression of proteins involved in cell cycle

regulation. Western blotting indicated that expression of the p27 protein (27 kDa), which is critical for cell cycle arrest in the G1 phase, was increased in *ECHDC1* knockdown cells compared to the control cells (Fig. 3).

## Discussion

iTRAQ proteomic analysis has been shown to be a useful technique for investigation of chemoresistant factors in cancer (6,10). We therefore used iTRAQ proteomic analysis



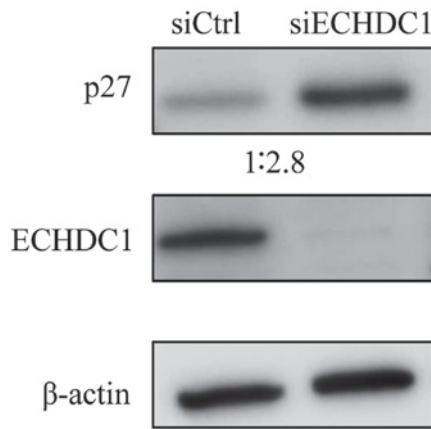


Figure 3. ECHDC1-siRNA decreases ECHDC1 protein expression. UMUC3GR cells were transfected with control or ECHDC1-siRNA (siCtrl and siECHDC1, respectively) and the ECHDC1 expression level was determined using western blotting.  $\beta$ -actin was used as a loading control. ECHDC1 expression level was clearly decreased in the UMUC3GR cells transfected with ECHDC1-siRNA. The expression level of p27 in cells transfected with ECHDC1- or control-siRNA (siECHDC1 or siCtrl, respectively) was determined using western blotting. Quantification of the western blot signal for p27 is shown at the top. p27 expression level was clearly increased in cells transfected with ECHDC1-siRNA compared with cells transfected with control-siRNA. The experiment was repeated three times.

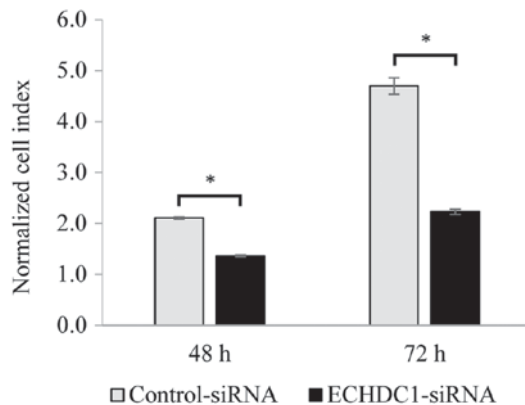


Figure 4. Effect of ECHDC1 silencing on the proliferation of UMUC3GR cells. The normalized cell index of UMUC3GR cells transfected with ECHDC1-siRNA (black bars) was significantly lower than that of cells transfected with control-siRNA (gray bars) at 48 and 72 h after transfection ( $n=4$ ). Values are expressed as means  $\pm$  standard deviation.  $*P<0.05$ . The experiment was repeated three times.

to identify proteins associated with gemcitabine resistance by comparing the expression of proteins in two gemcitabine-resistant cell lines (UMUC3GR, HT1376GR) with that of the two parental gemcitabine-sensitive cell lines (UMUC3, HT1376). This analysis showed that expression of the ECHDC1 protein was significantly increased in both of the gemcitabine-resistant cell lines compared to the parental cells.

ECHDC1 has been identified as a new metabolite proof-reading enzyme, ethylmalonyl-CoA decarboxylase (11,12). It is localized mainly in the cytosol and corrects a side activity of acetyl-CoA carboxylase. Acetyl-CoA carboxylase synthesizes malonyl-CoA from acetyl-CoA, and malonyl-CoA then feeds into the de novo fatty acid synthesis pathway (13). However, Acetyl-CoA carboxylase displays

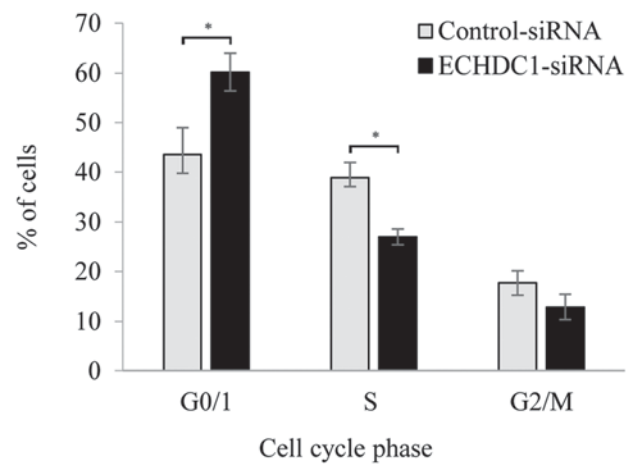


Figure 5. Effect of ECHDC1-siRNA on the cell cycle. Cell cycle analysis was performed on UMUC3GR cells at 48 h after transfection of control-siRNA or ECHDC1-siRNA. Data are expressed as a percent of the total cells. The experiment was repeated three times. Values are expressed as means  $\pm$  standard deviation  $*P<0.05$ .

a lack of substrate specificity, and it is also able to synthesize methylmalonyl-CoA and ethylmalonyl-CoA (14-16). Ethylmalonyl-CoA could perturb lipid synthesis by trapping CoA and inhibiting fatty acid synthesis, leading to the formation of abnormal ethyl-branched fatty acids due to its structural similarity with malonyl-CoA. Ethylmalonyl-CoA decarboxylase (ECHDC1) can eliminate ethylmalonyl-CoA by converting it to butyryl-CoA (11).

We observed that silencing of *ECHDC1* significantly inhibited bladder cancer cell proliferation. This is the first report to identify a function for ECHDC1 in cancer. The *ECHDC1* gene is included in a novel breast cancer risk locus on 6q22.33 that was identified in a genome-wide association study (17). However, the mechanism of induced cancer risk is unknown. In human cells, silencing of *ECHDC1* decreased ethylmalonyl-CoA decarboxylase activity and increased the formation of ethylmalonic acid (EMA) (11). EMA induces oxidative stress in skeletal muscle and in the cerebral cortex (18,19). Human cancer cells are more sensitive to oxidative stress, which inhibits cell proliferation (20). Oxidative stress regulates the intracellular level of p27 (21). The p27 protein is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors that bind to cyclin/CDK complexes and inhibit their activities. p27 arrests the cell cycle in the G1 phase (22-24). In agreement with these reports, we observed increased p27 expression and G1 arrest in bladder cancer cells in which *ECHDC1* was silenced.

The limitation of this study is that we could not identify the detailed mechanism by which gemcitabine increased ECHDC1 and by which reduction of ECHDC1 inhibited the growth of bladder cancer cells. Accumulation of EMA or perturbation of lipid synthesis by decreasing ethylmalonyl-CoA decarboxylase activity may be the cause. Further studies including animal or clinical specimens are required to understand the role of ECHDC1 in cancer.

In conclusion, ECHDC1 was increased in gemcitabine-resistant bladder cancer cells and silencing of *ECHDC1* inhibited cell proliferation. The present study suggested that

gemcitabine may have induced *ECHDC1* expression and that *ECHDC1* may be a novel potential target for development of gemcitabine-resistant bladder cancer treatment.

This article does not contain any studies with human participants or animals performed by any of the authors.

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