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Biology of Human Tumors

Clinical Cancer Research

ALDH1A3, the Major Aldehyde Dehydrogenase Isoform in Human Cholangiocarcinoma Cells, Affects Prognosis and Gemcitabine Resistance in Cholangiocarcinoma Patients

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Abstract

Purpose: Intrahepatic cholangiocarcinoma is a fatal primary liver cancer resulting from diagnosis at an advanced stage. Understanding the mechanisms of drug resistance and metastasis of cholangiocarcinoma may improve the disease prognosis. Enhanced aldehyde dehydrogenase (ALDH) activity is suggested to be associated with increased drug resistance and the metastasis. This study aims to investigate the roles of the ALDH isoforms in cholangiocarcinoma.

Experimental Design: Aldefluor assays, RT-PCR, and Western blot analysis were used to identify the major ALDH isoforms contributing to Aldefluor activity in human cholangiocarcinoma cell lines. We manipulated isoform expression in HuCCT1 cells to elucidate the role of ALDH1A3 in the malignant progression of these cells. Finally, we used immunohistochemical staining to evaluate the clinical significance of ALDH1A3 in 77 hepatectomized cholangiocarcinoma patients and an additional 31 patients with advanced cholangiocarcinoma who received gemcitabine-based therapy.

Results: ALDH^{high} cholangiocarcinoma cells not only migrated faster but were more resistant to gemcitabine. Among the 19 ALDH isoforms studied, ALDH1A3 was found to be the main contributor to Aldefluor activity. In addition, we also found that knockdown of ALDH1A3 expression in HuCCT1 cells markedly reduced not only their sensitivity to gemcitabine, which might be attributed to a decreased expression of ribonucleotide reductase M1, but also their migration. Most importantly, this enzyme was also identified as an independent poor prognostic factor for patients with intrahepatic cholangiocarcinoma, as well as a prognostic biomarker of gemcitabine-treated patients.

Conclusions: ALDH1A3 plays an important role in enhancing malignant behavior of cholangiocarcinoma and serves as a new therapeutic target. *Clin Cancer Res; 22(16); 4225–35. ©2016 AACR.*

Introduction

Although cholangiocarcinoma is a relatively rare hepatobiliary cancer, the incidence and mortality of cholangiocarcinoma have increased worldwide in recent years (1–3). In fact, intrahepatic cholangiocarcinoma is the second most common liver cancer, accounting for 10% to 15% of all primary liver malignancies (4). Surgical resection is the only available curative therapy for patients with intrahepatic cholangiocarcinoma. However, as most cases of cholangiocarcinoma are diagnosed at advanced disease stages when liver functions are already poor, palliative chemotherapy with gemcitabine and cisplatin is often the best treatment option (4, 5). Furthermore, local or distant recurrences frequently occur in patients who have undergone surgical resection (6); thus, a better understanding of the mechanisms underlying drug resistance and metastasis of cholangiocarcinoma may improve the prognosis of patients with this malignancy.

In humans, the aldehyde dehydrogenase (ALDH) superfamily of enzymes consists of 19 isoforms that are responsible for converting both exogenous and endogenous aldehydes to carboxylic acids (7, 8), and metabolism of the latter represents a major detoxification pathway in the body (7, 8). Different ALDH isoforms are used to identify and isolate neural stem cells (SC; ref. 9), human hematopoietic SCs (10), myogenic precursor cells (11), and adipose-derived adult SCs (12). Elevated ALDH activity is also observed in leukemic SC populations in some acute myelogenous leukemia patients (13). ALDH1 serves as a marker of cancer SCs (CSC) in several types

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Translational Relevance

Intrahepatic cholangiocarcinoma is a fatal primary liver cancer resulting from diagnosis at an advanced stage. Understanding the mechanisms of drug resistance and metastasis of cholangiocarcinoma may improve the disease prognosis. We demonstrated that ALDH^{high} cholangiocarcinoma cells not only migrated faster but were more resistant to gemcitabine. Among the 19 ALDH isoforms studied, ALDH1A3 was found to be the main contributor to Aldefluor activity. In addition, we also found that knockdown of ALDH1A3 expression in HuCCT1 cells markedly reduced not only their sensitivity to gemcitabine, which might be attributed to a decreased expression of ribonucleotide reductase M1, but also their migration. Most importantly, this enzyme was also identified as an independent poor prognostic factor for patients with intrahepatic cholangiocarcinoma, as well as a prognostic biomarker of gemcitabine-treated patients. ALDH1A3 plays an important role in enhancing malignant behavior of cholangiocarcinoma and serves as a new therapeutic target.

of solid tumors, including those of the liver (14), head and neck (15), pancreas (16), lung (17, 18), prostate (19), bladder (20), ovary (21), breast (22), and colon (23). On the other hand, the potential of using increased ALDH activities to identify SC subpopulations in cholangiocarcinoma remains to be verified (24). Furthermore, delineating the ALDH isoform(s) responsible for elevated enzyme activity in cholangiocarcinoma cells, as well as the correlations between ALDH upregulation and chemosensitivity of these cells to various agents, may be crucial for prognostic purposes.

In this study, we first demonstrated that ALDH^{high} HuCCT1 and SNU1079 human cholangiocarcinoma cells showed greater migration and gemcitabine resistance than ALDH^{low} cells. We next found that ALDH1A3 was the major isoform in the ALDH^{high} subpopulations of the aforementioned two human cholangiocarcinoma lines, and knockdown of its expression in HuCCT1 cells markedly reduced their migration and gemcitabine resistance. Data from our subsequent analyses suggested that the aforementioned effects of ALDH1A3 silencing in HuCCT1 cells could be attributed, respectively, to a mesenchymal–epithelial transition (MET) and a downregulated expression of ribonucleotide reductase M1 (RRM1). Finally, we demonstrated for the first time that higher ALDH1A3 expression was not only a poor prognostic factor for patients with intrahepatic cholangiocarcinoma, but also a prognostic factor for diminished responses to chemotherapy in cholangiocarcinoma patients. Taken together, our findings suggest an important role for ALDH1A3 in the malignant behaviors of human CAA.

Materials and Methods

Cell culture

HuCCT1 (purchased from the Japanese Collection of Research Bioresources) and SNU1079 (obtained from the Korean Cell Line Bank) cholangiocarcinoma cells were grown in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Biological Industries) and 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 mg/mL amphotericin B (PSA; Biological Industries). All cells were cultured at 37°C in a 5% CO_2 incubator.

Preparation of recombinant lentiviruses and establishment of stable cholangiocarcinoma clones

The pLKO.1-shALDH1A3 (TRCN0000414303) and pLKO.1-shLacZ (TRCN0000072224) vectors were purchased from the National RNAi Core Facility Platform (*Academia Sinica*). To prepare the desired lentiviruses, 293T cells (8×10^5) were seeded onto 6-cm dishes overnight and cotransfected with 2.25 μg of pCMV-ΔR8.91, 0.25 μg of pMD.G, and 2.5 μg of pLKO.1, using the PolyJet DNA In Vitro Transfection Reagent (SignaGen Laboratories). One day after transfection, cells were switched to a serum-free DMEM supplemented with 1% BSA, and lentiviral particles were collected, respectively, at 24 and 48 hours post-medium change. The two sequential collections were then mixed, and the relative infection unit of each pseudotyped lentivirus was determined by cell viability assays using A549 human lung adenocarcinoma cells as hosts. To establish stable clones expressing short hairpin RNAs (shRNA) against LAcZ or ALDH1A3, HuCCT1 cells were infected with lentiviral particles (multiplicity of infection = 5) carrying the appropriate DNA fragments for 24 hours, followed by puromycin (1 μg/mL) selection.

Aldefluor assay and FACS

Cholangiocarcinoma cells were trypsinized, pelleted, and resuspended in the assay buffer (STEMCELL Technologies) at a density of 1×10^6 cells/mL. Then, these cells were incubated with the substrate, BODIPY-aminoacetaldehyde, in the presence or absence of the ALDH inhibitor, N,N-diethylaminobenzaldehyde (DEAB), for 30 minutes at 37°C before being subjected to flow cytometry. The fluorescence of DEAB-treated cells, measured by a BD FACSCalibur, was used as the background, and cells with top 10% (HuCCT1) or 20% (SNU1079) fluorescence intensity were defined as ALDH^{high} subpopulation, whereas cells with lowest 10% (HuCCT1) or 20% (SNU1079) fluorescence were deemed as ALDH^{low} subpopulations. For sorting the ALDH^{high} and ALDH^{low} subpopulations, a BD FACSAria (Becton Dickinson) was applied.

Quantitative reverse transcription PCR

Reverse transcription was performed using 5 μg total RNAs isolated from cells and a reverse transcriptase (Fermentas Life Sciences) in the presence of both random hexamers and oligo-dT (Genomics BioSci & Tech). To prepare standards for quantifying the expression of ALDH isoforms in cholangiocarcinoma cells, fragments of 19 different ALDH isoforms were amplified with their respective isoform-specific primer sets, cloned into the pGEM-T easy vector (Promega), and sequenced. After confirming the correctness of their sequences, they were used to establish standard curves for quantifying the copy numbers of each ALDH isoform in cholangiocarcinoma cells using SYBR Green–based real-time PCR. A PCR fragment of GAPDH was also cloned into the pGEM-T easy vector for establishing GAPDH standard curves. The expression levels of ALDH isoforms were expressed as mRNA copy numbers/1,000 GAPDH mRNA molecules. The cycling conditions for real-time PCR included an initial denaturation cycle at 95°C for 10 minutes; 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds,

and 72°C for 40 seconds; and a final extension step at 72°C for 10 minutes, followed by a melting-curve analysis.

Cell viability measurements

Parental, sorted cholangiocarcinoma cells or stable cholangiocarcinoma clones were seeded into 96-well plates in normal growth medium and gemcitabine or cisplatin was added at the indicated concentrations on the next day. Cell viability was determined by MTT assays (R&D Systems) 48-hour post-drug treatment following the manufacturer's instructions.

Transwell migration assays

Sorted cholangiocarcinoma cells or stable cholangiocarcinoma clones were trypsinized and resuspended in serum-free RPMI1640 medium, and adjusted to a density of 1.25×10^5 (HuCCT1) or 5×10^5 (SNU1079) cells/mL. Six hundred microliters of complete medium were placed in the bottom chamber, and 200 μL of cell suspensions described above were seeded into the top chamber. The cells were allowed to migrate for 6 hours and then were fixed with 1% formaldehyde, followed by staining with 0.005% crystal violet. Before being examined by light microscopy (Olympus IX70, 10× objective), cells remaining in the top chamber were removed by cotton swabs. Three random fields were photographed for each transwell. The average number of cells migrated per field were calculated using MetaMorph software (Universal Imaging Corporation) based on the results of three independent experiments.

Western blotting

Total lysates from sorted cholangiocarcinoma cells or stable cholangiocarcinoma clones were prepared using RIPA lysis buffer and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates (25 μg protein) were then separated by electrophoresis on 10% SDS–PAGE gels and transferred onto nitrocellulose membranes. The membranes were probed, respectively, with primary antibodies against ALDH1A3, RRM1, Oct4, Nanog, Bmi1, E-cadherin, N-cadherin, Twist, or β-actin at 4°C overnight. After washing with TBST buffer (25 mmol/L Tris, 137 mmol/L NaCl, 0.075% Tween 20; pH 7.4), the membranes were incubated with either a horseradish peroxidase (HRP)-conjugated anti-goat IgG (1:5,000; Sigma-Aldrich) or a HRP-conjugated anti-mouse IgG (1:3,000; GE Healthcare). The proteins were visualized using enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences, Inc.), according to the manufacturer's instructions. All antibodies used were purchased from GenTex International Corporation.

Patient demographics

We examined the demographic features of 77 patients with mass-forming cholangiocarcinoma (MF-CCA) who underwent hepatectomy between 1989 and 2006 at the Department of Surgery of Chang Gung Memorial Hospital as well as 31 patients with advanced (either nonresectable or recurrent/metastatic) intrahepatic cholangiocarcinoma who received 25 mg/m^2 cisplatin followed by 1,000 mg/m^2 gemcitabine by intravenous infusion on days 1 and 8 of each 21-day cycle (5). The study was approved by the local institutional review board of Chang Gung Memorial Hospital (clinical study numbers 99-2886B, 99-3810B, and 102-5813B), and written informed consent for drug administration and immunohistochemical tumor analysis was obtained from each patient.

ALDH1A3 IHC

ALDH1A3 expression levels in the aforementioned 77 MF-CCA patients plus 31 patients with advanced cholangiocarcinoma were examined by immunohistochemical staining. Tissue sections (4 μm) prepared from the formalin-fixed, paraffin-embedded hepatectomy specimens were incubated with the primary antibody against ADH1A3 (N2C2, 1:500 dilution; GeneTex) at 4°C overnight. After washing thrice with TBST (5 minutes each), the signals were visualized with the Dako Labeled Streptavidin-Biotin2 (LSAB2) System-HRP (Dako A/S, No. K0675; Dako). Control slides were incubated with the secondary antibody only. For the assessment of immunohistochemical staining, the percentage of stained target cells was evaluated in 10 random microscopic fields per tissue section (×400 magnification), and their averages were subsequently calculated. Staining intensities were scored as 1 (mild), 2 (moderate), or 3 (strong). H-scores were calculated as the percentage of positive staining (0–100) × the corresponding staining intensity (0–3). Specimens with H-scores of <100 or ≥100 were classified as having low or high expression, respectively (range: 5–295; median 100).

Statistical analysis

All data were presented as the mean ± SD. Differences between the experimental and control groups were calculated using the Student t test. Progression-free survival (PFS) and overall survival (OS) rates were evaluated using the Kaplan–Meier method. Several clinicopathologic variables were considered for the initial univariate analysis, which was performed using the log-rank test. The Cox proportional hazards model was applied for multivariate regression. The SPSS for Windows (Version 17.0, Chicago) was used for statistical analysis. A value of $P \leq 0.05$ derived from two-tailed test was considered statistically significant.

Results

Enhanced migration and increased resistance to gemcitabine, but not cisplatin are observed in ALDH^{high} cholangiocarcinoma cells

To confirm the presence of an ALDH^{high} subpopulation in cholangiocarcinoma cells, expression levels of this enzyme in two human intrahepatic cholangiocarcinoma cell lines, HuCCT1 and SNU1079, were analyzed by Aldefluor assays. As shown in Fig. 1, ALDH^{high} subpopulations were found in the HuCCT1 (∼10%) and SNU1079 (∼20%) cell lines; however, these subpopulations were not detected when DEAB, a commonly used selective ALDH inhibitor, was added. Transwell migration assays were then performed to compare the relative migration abilities of ALDH^{high} and ALDH^{low} cells and we found that the former migrated much faster than the latter (Fig. 1B). A well-known hallmark of CSCs is their resistance to conventional chemotherapeutic agents. Because gemcitabine and cisplatin are currently used as the first-line drugs for treating biliary tract cancer (5), the cytotoxic effects of these drugs on cholangiocarcinoma cells were examined. Interestingly, while the ALDH^{high} cells were more resistant to gemcitabine treatment than ALDH^{low} cells, no significant difference in the sensitivity to cisplatin of these two subpopulations was observed (Fig. 1C).

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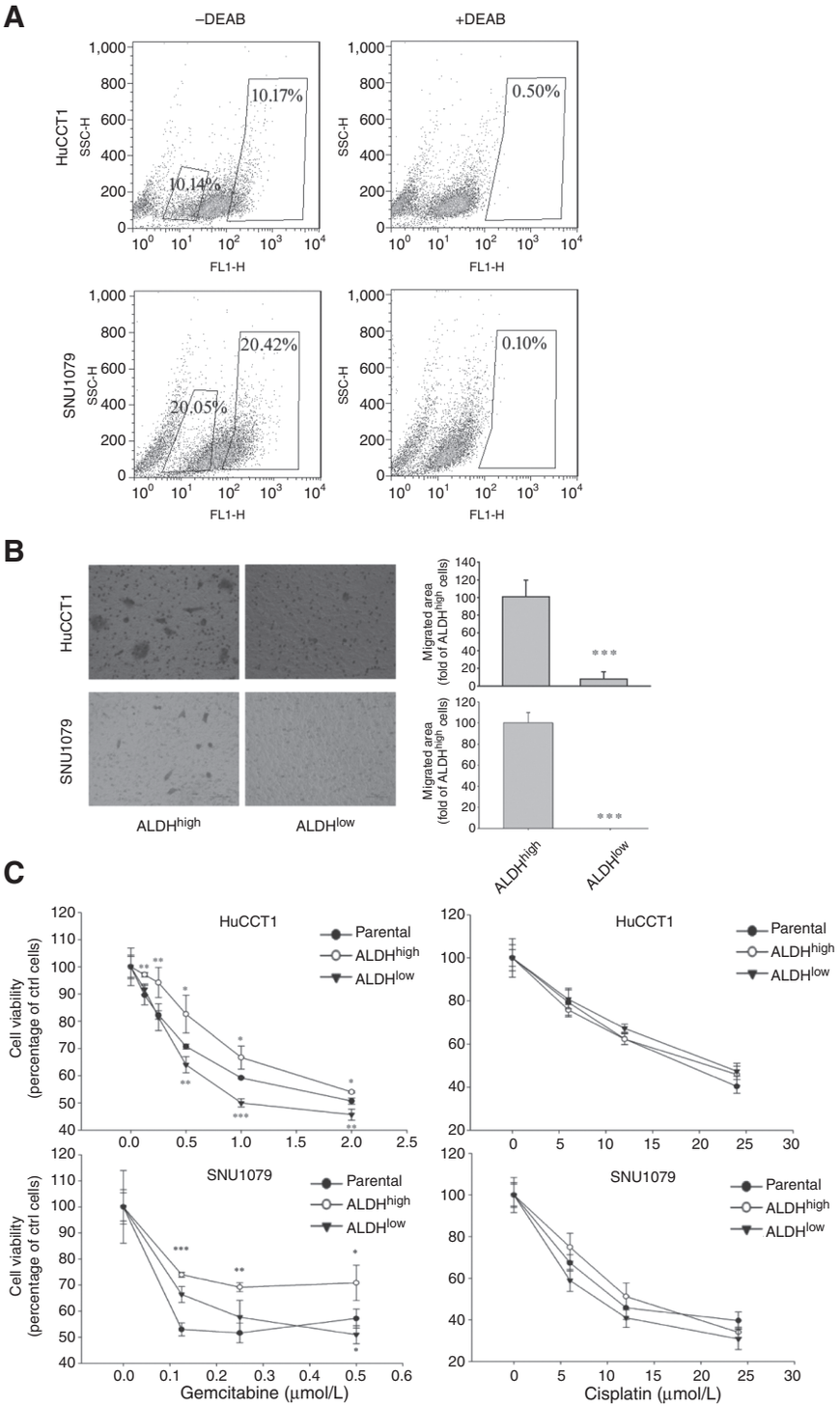

Figure 1.
ALDH^{high} cells isolated from two human cholangiocarcinoma lines have higher migration ability and increased drug resistance to gemcitabine, but not cisplatin. **A,** HuCCT1 and SNU1079 cholangiocarcinoma cells were trypsinized and the ALDH activities were measured by Aldefluor assay as per the manufacturer's instruction. ALDH-positive cells (left) were identified by comparing with profile of Aldefluor fluorescence of cells treated with ALDH inhibitor, DEAB (right). ALDH^{high} and ALDH^{low} cells were defined as described in Materials and Methods and annotated. **B,** HuCCT1 and SNU1079 cholangiocarcinoma cells were labeled with Aldefluor fluorescent reagent, and then the ALDH^{high} and ALDH^{low} cells were sorted by FACS as described in Materials and Methods. The sorted cells were then cultured in normal culture medium for another 2 days before being subjected to transwell migration assay. The cells were seeded and allowed to migrate for 6 hours before the nonmigrated cells remained on the upper side of the membrane were wiped out with cotton swab and the cells on the lower side were stained with crystal violet and counted as described in Materials and Methods. Data shown are the averages of three independent experiments. ***, $P < 0.001$ analyzed by Student t test. **C,** for analyzing the drug sensitivity of different subpopulations, 2×10^4 parental, ALDH^{high} or ALDH^{low} cholangiocarcinoma cells were seeded onto 96-well plates and after overnight incubation various amounts of gemcitabine (left) or cisplatin (right) were added. Forty-eight hours after drug treatment, cell viability was measured by MTT assay and was normalized to the respective untreated cells. Data shown are the averages of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with the parental cells with the same treatments analyzed by Student t test.

ALDH1A3 is primarily responsible for ALDH activity in cholangiocarcinoma cells

To determine which ALDH isoform(s) accounted for most of the ALDH activity detected in the two cholangiocarcinoma cell lines, RT-qPCR was performed to measure the mRNA levels of each ALDH isoform. As shown in Fig. 2A, increased mRNA expression of the ALDH isoforms 1A3 and 1L1 was detected in HuCCT1 cells, whereas increased expression of the 1A3, 1B1, 6A1, 1A1, 18A1, 3B2, and 3B1 isoforms was detected in SNU1079 cells. Among these ALDH isoforms, ALDH1A3 exhibited the most significant and consistent increase in the ALDH^{high} subpopulations of both

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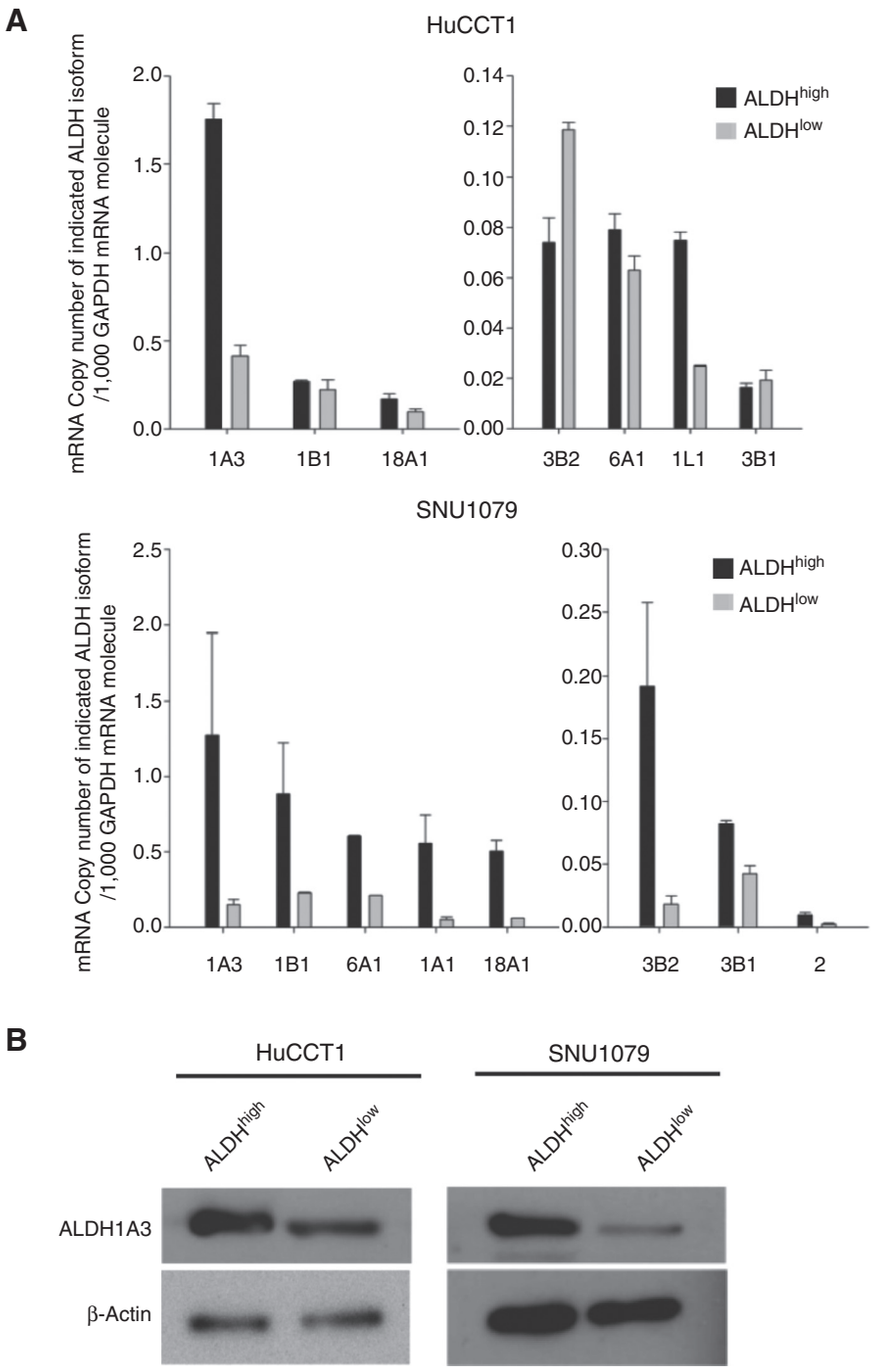

Figure 2.
ALDH1A3 is mainly responsible for the ALDH activity in two human cholangiocarcinoma lines. **A,** immediately after FACS sorting, both ALDH^{high} and ALDH^{low} HuCCT1 and SNU1079 cells were lysed by TRIzol and the total RNAs were harvested for reverse transcription. The amounts of mRNA of indicated ALDH isoforms and GAPDH were then analyzed by quantitative PCR with standard curves. Data are expressed as copy numbers of the indicated ALDH isoforms per 1,000 GAPDH mRNA molecules. **B,** total lysates (25 μg) from the aforementioned cholangiocarcinoma cells were subjected to Western blot analysis using an anti-ALDH1A3 antibody as a probe. β-Actin signals were used as loading controls.

cholangiocarcinoma cell types, suggesting that this enzyme might be largely responsible for the ALDH activity detected by the Aldefluor assays. Western blot analysis was then performed to analyze ALDH1A3 protein levels in the ALDH^{high} and ALDH^{low} subpopulations of both HuCCT1 and SNU1079 cells. As expected, ALDH1A3 protein levels were much higher in the ALDH^{high} subpopulations of these two cholangiocarcinoma lines (Fig. 2B).

ALDH1A3 knockdown in HuCCT1 cells increases their sensitivity to gemcitabine, which may be accounted by a downregulated expression of ribonucleotide reductase subunit M1

To further evaluate the role of ALDH1A3 in cholangiocarcinoma malignancy, we established ALDH1A3-knockdown HuCCT1 cells (Fig. 3A) and found that they were more sensitive to gemcitabine than the lacZ-knockdown cells (Fig. 3B).

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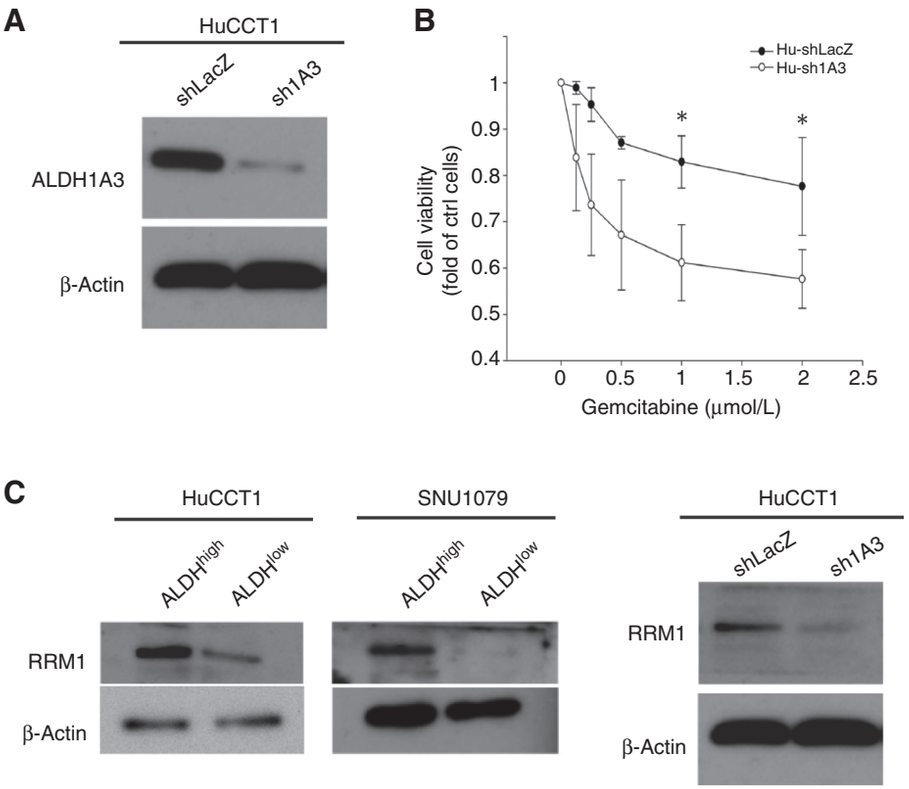

Figure 3.
ALDH1A3 confers the gemcitabine resistance to HuCCT1 cells. **A,** stable HuCCT1 cells expressing shRNAs against LacZ (shLacZ) and ALDH1A3 (sh1A3), respectively, were established after recombinant lentivirus infections and puromycin selection. Western blotting was then performed to confirm the success of ALDH1A3 knockdown. **B,** drug sensitivities of the LacZ- and ALDH1A3-knockdown cells were analyzed by MTT-based viability assays 48 hours after they were treated with indicated concentrations of gemcitabine. Cell viability was normalized to the untreated LacZ-knockdown cells and the data shown are the averages of three independent experiments. *, $P < 0.05$ analyzed by Student *t* test. **C,** total lysates (25 μg) prepared, respectively, from the ALDH^{high} and ALDH^{low} HuCCT1 (left) and SNU1079 (central) cells as well as from the LacZ- and ALDH1A3-knockdown HuCCT1 cells (right) were subjected to Western blot analyses using an antibody against RRM1. β-Actin signals were used as loading controls.

Interestingly, downregulated expression of RRM1, the main metabolizing enzyme for gemcitabine (25), was detected not only in ALDH1^{low} HuCCT1 and SNU1079 cells but also in ALDH1A3-knockdown HuCCT1 cells (Fig. 3C), which might explain, at least partially, the increased sensitivity of these cells to gemcitabine.

ALDH1A3 knockdown diminishes the migration ability of HuCCT1 cells possibly by inducing a MET

As Shuang and colleagues demonstrated in a recent study that TGFβ1 induced an epithelial–mesenchymal transition (EMT) in another human cholangiocarcinoma line, TFK-1, which was accompanied with increased ALDH1 expression (24), we examined whether altering the expression levels of ALDH1A3 in HuCCT1 cells affected their EMT phenotype. Indeed, we found that ALDH1A3 knockdown in these cells drastically decreased their migration ability (Fig. 4A), which was associated with significant reductions of Bmi-1 and Twist expression, but a marked increase of E-cadherin, the best-known epithelial marker (Fig. 4B). However, this treatment did not influence the expression levels of Oct4 and Nanog, two embryonic SC markers, in HuCCT1 cells. Together, these results suggested that downregulation of ALDH1A3 might induce MET in human cholangiocarcinoma cells.

Correlations between ALDH1A3 expression and clinicopathologic features in patients with MF-CCA who underwent hepatectomy and in those with advanced intrahepatic cholangiocarcinoma receiving chemotherapy

We next analyzed correlations between ALDH1A3 expression levels and clinicopathologic features in two groups of cholangiocarcinoma patients. ALDH1A3 is diffusely expressed in the cytoplasm in MF-CCA (Fig. 5A). Among specimens from 77 MF-CCA patients who underwent hepatectomy, 38 (49.4%) showed strong ALDH1A3 staining intensity (e.g., H-scores $\geq$100). Interestingly, ALDH1A3 upregulation strongly correlated with elevated alkaline phosphatase (ALP) levels and a positive resection margin. However, only the latter was independently associated with ALDH1A3 overexpression (Supplementary Table S1). In addition, a significant positive association between ALDH1A3 upregulation and disease progression was detected in 31 advanced intrahepatic cholangiocarcinoma patients receiving gemcitabine-based chemotherapy (Supplementary Table S2).

ALDH1A3 may be a poor prognostic indicator for MF-CCA patients who underwent hepatectomy and in those with advanced intrahepatic cholangiocarcinoma receiving gemcitabine-based chemotherapy

Univariate log-rank analysis was then applied to identify factors that had adverse influences on the OS rate in the aforementioned MF-CCA patients. The presence of disease symptoms (elevated ALP and CEA levels, a tumor size > 5 cm, positive surgical-margin status) and strong ALDH1A3 staining intensity were found to correlate with OS (Table 1). However, only a tumor size > 5 cm, positive surgical-margin and strong ALDH1A3 staining intensity were identified as independent predictors for an inferior OS rate in these patients after multivariate Cox proportional hazard analysis (Table 1 and Fig. 5B). In the meantime, for patients with advanced intrahepatic cholangiocarcinoma receiving chemotherapy, high ALDH1A3 expression was correlated with inferior PFS,

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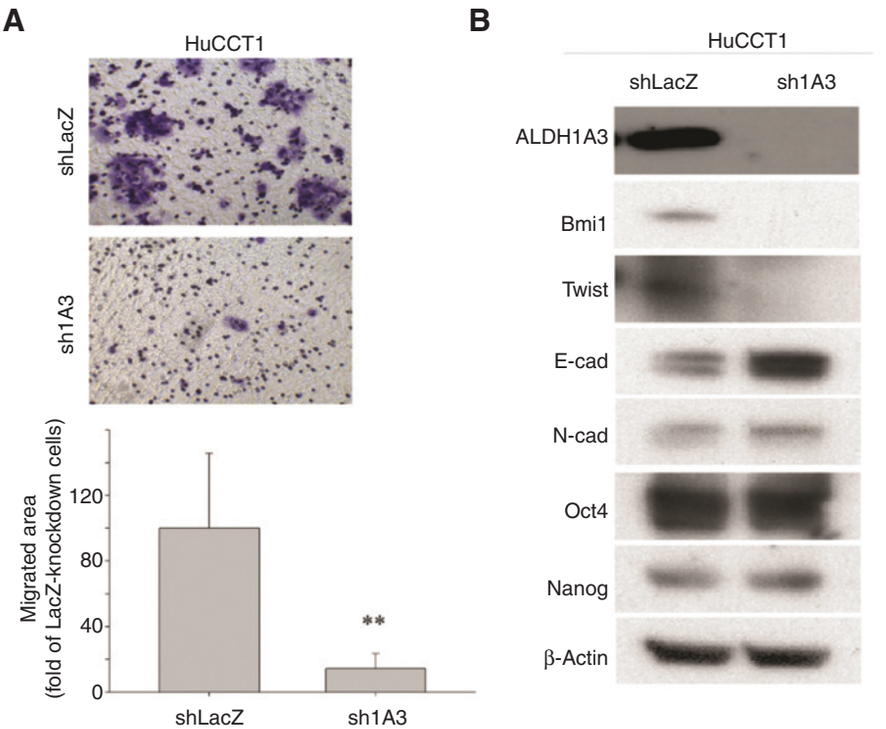

Figure 4.
ALDH1A3-knockdown expression in HuCCT1 cells reduces their migration ability which may be accounted by a MET transition. **A,** the migration abilities of the LacZ- and ALDH1A3-knockdown HuCCT1 cells were analyzed by transwell migration assays as above described. Data (left, bottom) shown are the averages of three independent experiments. **, $P < 0.01$ when compared with the LacZ-knockdown cells by Student *t* test. **B,** total lysates (25 μg) prepared, respectively, from the LacZ- and ALDH1A3-knockdown HuCCT1 cells were subjected to Western blot analyses using antibodies against a variety of EMT markers (i.e., Twist, E-cadherin, N-cadherin, and Bmi1) as well as several embryonic SC markers (i.e., Oct4 and Nanog). β-Actin signals were used as loading controls.

OS, and objective response rates (Supplementary Table S2; Fig. 5C and D). These observations indicated that low ALDH1A3 expression might be also a useful prognostic factor for positive responses to chemotherapy in cholangiocarcinoma patients.

Discussion

In the current study, we isolated ALDH^{high} cells from two intrahepatic cholangiocarcinoma cell lines, HuCCT1 and SNU1079, and determined that ALDH1A3 is the main contributor to ALDH activity. Previous studies have reported that ALDH activities measured by the Aldefluor assay may differ among different types of cancer. For example, van den Hoogen and colleagues found that ALDH7A1 is highly expressed in prostate cancer cell lines, prostate cancer tissues, and bone metastasis samples, suggesting that ALDH7A1 is responsible for ALDH activity in prostate cancer (19). In another study, Chen and colleagues compared the levels of ALDH1A1 and ALDH1B1 in various adenocarcinomas (colon, lung, breast, and ovary) by immunohistochemical staining and reported a 5.6-fold higher expression of ALDH1B1 than ALDH1A1 in these cancerous tissues. In addition, they also found that ALDH1B1 is dramatically upregulated in human colonic adenocarcinoma and therefore considering it as a potential biomarker for human colon cancer (23). Although high ALDH1A3 levels have already been detected in more aggressive forms of breast, bladder, and lung cancers (18, 22, 26), this work is nevertheless the first, to our knowledge, to report that ALDH1A3 is the main ALDH isoenzyme in intrahepatic cholangiocarcinoma. More importantly, we also found that ALDH1A3 is a critical prognostic factor in 77 patients with intrahepatic cholangiocarcinoma who received hepatectomies among several clinicopathologic features. (Table 1; Fig. 5B). However, the smaller patient number might be the limitation of survival analysis in this study. In addition, we showed that ALDH1A3 is also a prognostic biomarker of gemcitabine-treated patients using samples from 31 patients with advanced cholangiocarcinoma as a validation cohort (Fig. 5C and D).

ALDH activity is a universal CSC marker (7), and most studies report that drug resistance induced by ALDH upregulation is frequently due to the increase of CSC population (7). CSCs are slow-growing cells which are generally quiescent and therefore are resistant to drugs that target fast-growing cancer cells (27). Another possible resistance mechanism resulted from ALDH upregulation can be attributed to an increased ALDH-mediated metabolism of drugs such as cyclophosphamide (28). As treatment with gemcitabine and cisplatin is currently the standard therapy for treating cholangiocarcinoma (5), we investigated whether ALDH1A3 expression in human cholangiocarcinoma cells affects their responses to these drugs. To our surprise, ALDH1A3 expression conferred resistance to gemcitabine, but not to cisplatin in two human cholangiocarcinoma lines (Fig. 1C), which cannot be explained simply by the quiescent properties of CSCs because both drugs target the rapid-growing cancer cells. Gemcitabine, a pyrimidine-based antimetabolite, needs to be converted into gemcitabine triphosphate before being incorporated into DNA to inhibit DNA synthesis and induce apoptosis (29). In this regard, RRM1 is a molecular target of gemcitabine which plays a key role in gemcitabine resistance (25). Results from previous studies not only showed that increased RRM1 expression occurs after the continuous exposure of non–small cell lung cancer (NSCLC) cells to gemcitabine but also led to the postulation that the survival of NSCLC patients with low RRM1 expression might be improved by gemcitabine treatment (30, 31). In our

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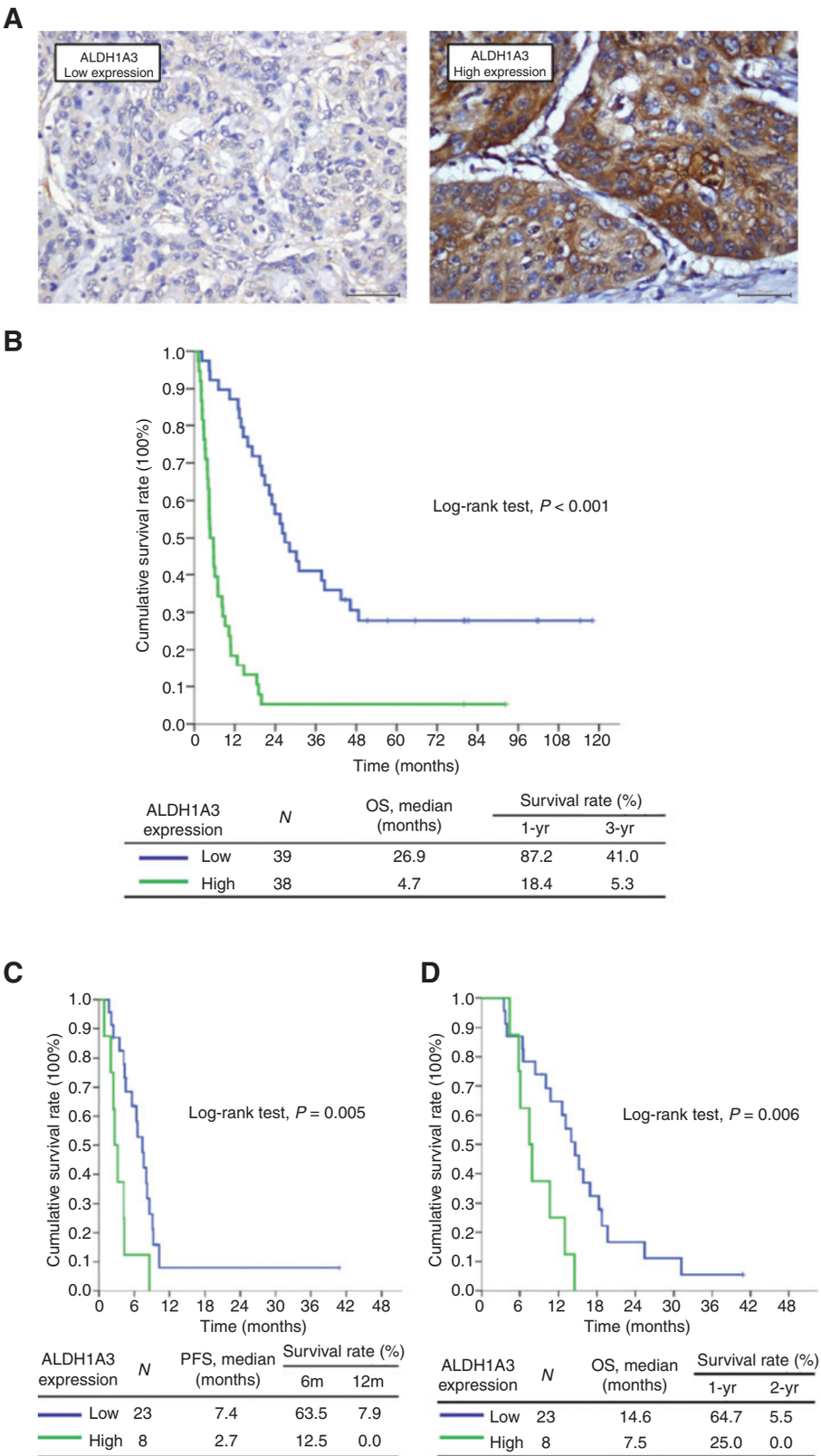

Figure 5.
ALDH1A3 expression levels are inversely correlated with the survival of patients with resectable mass-forming and advanced cholangiocarcinoma, respectively. **A,** pictures on the left and right show low and high ALDH1A3 immunohistochemical staining intensities, respectively. Scale bar, 50 μm. **B,** Kaplan–Meier plot of OS in patients with resectable MF-CCA tumors based on their ALDH1A3 expression levels. **C,** the ALDH1A3 group with high H-scores showed poorer OS in patients with resectable cholangiocarcinoma ($P = 0.001$). Kaplan–Meier plots of PFS (**C**) and OS (**D**) of 31 patients with advanced cholangiocarcinoma according to their ALDH1A3 expression levels.

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Table 1. Univariate and multivariate analysis of factors influencing the OS of MF-CCA patients

Factors	Median survival time (months)	95% CI of median	Univariate *P* value	Relative risk (95% CI)	Multivariate *P* value
Gender			0.757		
Male ($n = 33$)	14.70	7.89–21.50			
Female ($n = 44$)	10.46	4.97–15.94			
Age			0.473		
≤60 ($n = 37$)	13.84	3.46–24.22			
>60 ($n = 40$)	12.99	7.23–18.74			
Symptoms			0.007	2.338 (0.973–5.620)	0.058
Negative ($n = 12$)	37.71	4.78–70.63			
Positive ($n = 65$)	10.46	5.41–15.50			
AST (IU/L)			0.180		
≤34 ($n = 38$)	13.32	8.64–17.98			
>34 ($n = 38$)	10.72	2.28–19.16			
ALT (IU/L)			0.661		
≤36 ($n = 40$)	12.99	6.41–19.56			
>36 ($n = 30$)	14.70	6.23–23.17			
ALP (IU/L)			0.011	0.788 (0.392–1.583)	0.503
≤94 ($n = 23$)	23.90	11.65–36.15			
>94 ($n = 51$)	9.11	5.39–12.82			
Bil (total; mg/dL)			0.567		
≤1.3 ($n = 63$)	13.32	5.86–20.77			
>1.3 ($n = 14$)	10.72	0.00–22.23			
Albumin (g/dL)			0.063		
≤3.5 ($n = 22$)	4.70	3.11–6.29			
>3.5 ($n = 49$)	19.04	13.31–24.76			
Serum CEA (ng/dL)			0.052		
≤5 ($n = 25$)	18.51	2.09–34.93			
>5 ($n = 30$)	9.11	4.12–14.10			
Margin			<0.001	2.019 (1.076–3.790)	0.029
Negative ($n = 56$)	19.04	13.93–24.14			
Positive ($n = 21$)	4.70	2.24–7.16			
Size			0.006	1.948 (1.083–3.502)	0.026
≤5 cm ($n = 32$)	19.99	13.75–26.23			
>5 cm ($n = 42$)	8.45	2.39–14.51			
Lymph node			0.071		
Negative ($n = 47$)	19.89	16.71–23.07			
Positive ($n = 28$)	10.29	1.13–19.45			
Histology			0.215		
Well ($n = 2$)	2.73	NA			
Moderate ($n = 41$)	13.84	7.45–20.23			
Poor ($n = 32$)	12.99	3.46–22.51			
Others ($n = 2$)	4.37	NA			
ALDH1A3 expression			<0.001	6.151 (3.176–11.915)	<0.001
Low expression ($n = 39$)	26.86	19.06–34.67			
High expression ($n = 38$)	4.70	2.67–6.74			
Hepatitis B			0.259		
Negative ($n = 49$)	13.32	7.86–18.77			
Positive ($n = 16$)	19.99	10.01–29.91			

Abbreviation: CI, confidence interval.

study, significant decreases in the RRM1 levels were found in ALDH1^{low} HuCCT1 and SNU1079 cells as well as in ALDH1A3-knockdown HuCCT1 cells (Fig. 3C), which may at least in part explain their increased sensitivity to gemcitabine (Figs. 1C and 3). In good agreement, when the correlation between ALDH1A3 expression levels and response to chemotherapy in 31 cholangiocarcinoma patients who had received gemcitabine-based chemotherapy were examined, higher response rate was also detected in patients that expressed low levels of ALDH1A3 (Supplementary Table S2).

EMT is a key event for the increases in both cancer invasiveness and CSC properties (32). Shuang and colleagues discovered that TGFβ1 induced EMT together with the appearance of ALDH1-positive cells in cholangiocarcinoma (24). In accordance, a reduction in mesencymal properties ofALDH1A3-knockdown cells was found (Fig. 4), suggesting that ALDH1A3 upregulation may increase the migration ability of HuCCT1 cells via inducing an EMT.

In conclusion, we discovered that ALDH1A3 is the main contributor to Aldefluor activity in both HuCCT1 and SNU1079 cholangiocarcinoma cells. We also demonstrated that ALDH1A3 expression levels in these cells are positively correlated with their resistance to gemcitabine and knockdown of ALDH1A3 in HuCCT1 cells markedly increases their drug sensitivity and severely decreases their migration ability possibly by reducing RRM1 expression and mesencymal phenotypes. Most importantly, we demonstrated for the first time that ALDH1A3 is a poor prognostic factor for patients with intrahepatic cholangiocarcinoma who underwent hepatectomy and in those with advanced intrahepatic

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cholangiocarcinoma receiving chemotherapy. Collectively, our results suggest ALDH1A3 as a new therapeutic target for cholangiocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.-J. Weng, C.-T. Cheng, S.-C. Huang, C.-E. Wu, Y.-H. Chung, Y. Su, C.-N. Yeh

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-H. Chen, J.-J. Weng, C.-T. Cheng, Y.-H. Chung, C.-Y. Liu, M.-H. Chen, Y. Su, C.-N. Yeh

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ALDH1A3, the Major Aldehyde Dehydrogenase Isoform in Human Cholangiocarcinoma Cells, Affects Prognosis and Gemcitabine Resistance in Cholangiocarcinoma Patients

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