Suppressive expression of CD274 increases tumorigenesis and cancer stem cell phenotypes in cholangiocarcinoma

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Cholangiocarcinoma is an aggressive malignant tumor originating from intrahepatic or extrahepatic bile ducts. Its malignant phenotypes may be assumed by cancer stem cells (CSC). Here, we demonstrate that CD274 (PD-L1), known as an immunomodulatory ligand, has suppressive effects on CSC-related phenotypes of cholangiocarcinoma. Using two human cholangiocarcinoma cell lines, RBE and HuCCT1, we attempted to isolate the CD274^{low} and CD274^{high} cells from each cell line, and xenografted them into immunodeficient NOD/scid/γcnull (NOG) mice. We found that the CD274^{low} cells isolated from both RBE and HuCCT1 are highly tumorigenic in NOG mice compared with CD274^{high} cells. Furthermore, the CD274^{low} cells possess several CSC-related characteristics, such as high aldehyde dehydrogenase (ALDH) activity, reduced reactive oxygen species production and a dormant state in the cell cycle. Furthermore, depletion of CD274 expression by shRNA in RBE cells enhances their tumorigenicity and increases ALDH activity. These findings are compatible with our observation that clinical cholangiocarcinoma specimens are classified into low and high groups for CD274 expression, and the CD274 low group shows poorer prognosis when compared with the CD274 high group. These results strongly suggest that CD274 has a novel function in the negative regulation of CSC-related phenotypes in human cholangiocarcinoma, which is distinct from its immunomodulatory actions.

Cholangiocarcinoma is the most common primary malignancy of the biliary tract and one of the most difficult intra-abdominal malignancies to treat.[1] Surgical management is the only potentially curative treatment, but it is limited to the early stage of disease. Although cholangiocarcinoma stem cells were defined in a chemically induced rat hepatocarcinoma[2] and the deregulated self-renewal of hepatic stem/progenitor cells is an early event in the carcinogenesis of cholangiocarcinoma,[3] little is known about the relationship between the role of cancer stem cells (CSC; also known as tumor-initiating cells) and the pathogenesis of human cholangiocarcinoma.

Accumulating evidence suggests that CSC play pertinent roles in the generation of various tumors.[4] The current consensus describes CSC as a certain type of cell within a tumor that possesses self-renewal potential and heterogeneity-forming capability, which consequently function to promote tumor formation. The CSC from developmentally diverse tumors and established tumor cell lines have been isolated by using cell-surface markers expressed specifically on the CSC and are commonly defined as being highly tumorigenic on injection into immunodeficient mice. The CSC in several cancers are known to have high aldehyde dehydrogenase (ALDH) activities and reduced levels of reactive oxygen species (ROS).[5,6] In contrast, CD13 is a marker of semiquiescent or dormant CSC in human liver cancer.[7] The CD13$^+$ CSC predominately reside in the G0 phase of the cell cycle and compared with their CD13$^-$ counterparts they exhibit less ROS-induced DNA damage, resulting in protection from apoptosis.[8] Similarly, in acute and chronic myeloid leukemia,[9] the CSC survive in the dormant G0 phase of the cell cycle in a bone marrow niche. These observations suggest a striking parallel between the CSC cell cycle status and tumor dormancy.

There are many reports on specific surface markers that contribute to CSC enrichment in a variety of cancers; breast cancer-initiating cells are CD44$^+$CD24$^-$,[10] liver cancer stem cells are CD90$^+$,[11] and CD133 is a marker of CSC in glioblastoma,[12] pancreatic cancer[13] and gastric cancer.[14] We also reported that CD271 is a marker of CSC in hypopharyngeal cancer and that high levels of CD271 expression in tumor tissues correlate with a poor prognosis.[15] However, little is

known about the surface marker of CSC in cholangiocarcinoma. Since CSC initiate the tumor-forming process, they are expected to provide optimal target(s) for therapy.

Recent evidence highlights the pivotal role of CD274, also known as PD-L1 (programmed cell death ligand 1) or B7-H1 (B7 homolog 1), in maintaining an immunosuppressive tumor microenvironment. Because CD274 is commonly upregulated on various types of tumors and its receptor, PD-1, is expressed on the majority of tumor-infiltrating lymphocytes including T cells, the tumor cells expressing CD274 are thought to induce suppression of local antitumor T-cell responses.[16] In contrast to this immunosuppressive effect, CD274 is also known to contribute to the promotion of tumor cell growth and the downregulation of quiescent cells in breast cancer,[17] and not to express exclusively in CD133-positive stem cells in glioma,[18] suggesting a possible involvement of CD274 on regulation of cancer stem cells. Here, we demonstrated that CD274 suppresses the CSC-related phenotypes and becomes a prognostic factor for patients with human cholangiocarcinoma.

Materials and Methods

Ethics statements. This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Ethics Committees at Tohoku University Graduate School of Medicine (Sendai, Japan) and Miyagi Cancer Center Research Institute (Natori, Japan). The protocol of animal experiments was approved by the Miyagi Cancer Center Animal Care and Use Committee (permit number: MCC-AE-2011-8).

Patients and tissue specimens. Tumor specimens were obtained from 91 consecutive cases of cholangiocarcinoma in which patients underwent R0 surgical resection, according to the TNM classification (7th edition) from 2000 to 2008 at the Department of Surgery at Tohoku University Hospital (Sendai, Japan). Cancer staging was performed according to the TNM classification (7th edition).[19] Written informed consent was obtained from each patient prior to surgery.

Cell culture and shRNA transfection. The human cholangiocarcinoma cell lines used here were the RBE and HuCCT1 lines (provided by RIKEN BioResource Center, Tsukuba, Japan). They were maintained in RPMI-1640 medium containing 10% fetal bovine serum and antibiotics. To express the CD274-specific short hairpin RNA (shRNA), a retroviral vector was generated as described previously.[20] The target sequences were: 5′-GAGGAAGACCTGAAGGTTCAGCATA-3′ (RNAi-1) and 5′-GCCTACTGGCATTTGCTGAACGCATT-3′ (RNAi-2).

Quantitative real-time PCR. mRNA levels were measured using quantitative real-time PCR as described previously.[15] The primer sequences are listed in Supporting Information Table S1.

Flow cytometry analysis. Cells were assessed for surface marker expression using fluorescent multicolor flow cytometry (FACSCanto II; Becton Dickinson, San Jose, CA, USA) as described previously.[21] The antibodies used were anti-human CD274 antibodies (Biolegend, San Diego, CA, USA) and anti-human CD133 antibody (Miltenyi Biotec, Gladbach, Germany).

Histology and immunohistochemistry. Anti-Ki-67 staining was performed on a Ventana Discovery automation system (Roche, Basel, Switzerland). For staining with anti-CD274 antibodies (ab58810, rabbit polyclonal antibody; Abcam,

Cambridge, MA, USA), antigen retrieval was carried out by heating in a microwave for 15 min in Dako Target Retrieval Solution, High pH (Dako, Glostrup, Denmark). The antigen–antibody complex was visualized with diaminobenzidine solution (DAB; Dako EnVision Kit, HRP; Dako, Glostrup, Denmark) and counterstained with hematoxylin. CD274 immunohistochemistry was scored as "±" when samples showed either no detectable CD274 or weak CD274 staining of tumor cells. A score of "++" represented moderate to strong staining of the tumor cells. The staining grade was defined according to the majority of the DAB staining intensity throughout a specimen.

Biochemical analyses. To quantify the ROS levels, the cells were incubated in 5 μM 2', 7'-Dichlorodihydrofluorescin diacetate (DCFH-DA) for 30 min at 37°C, washed twice with PBS, stimulated with 20 mM H_2O_2 for 20 min if required and then subjected to flow cytometric analysis. The ALDH activities were determined using the Aldefluor assay according to the manufacturer's protocol (Aldegen, Durham, NC, USA). Cell proliferation rates were measured using the 3-(4, 5-dimethylthial-2-yl)-2, 5-diphenyltetrazalium bromide assay. All assays were performed in triplicate.

Measurement of retinoic acid. Retinoic acid was quantified as previously reported,[22] with minor modifications. Briefly, cells were incubated with 0.5 μM retinal for 16 h. The cells and culture supernatant were harvested and mixed with ethanol and then with 4.25 M NaCl and 0.02 M HCl. The extracted fractions with hexane were evaporated and dissolved in ethanol. The retinoids were separated on a Wakosil-II3C18AR column (3 μm, 4.6 × 50 mm) (Wako Chemicals, Osaka, Japan) at 40°C. The chromatography equipment consisted of two Jasco 880-PU HPLC pumps (Jasco Co., Tokyo, Japan), a Jasco875-UV uv/vis detector (350 nm) and a Jasco 860-CO column oven. The elu-
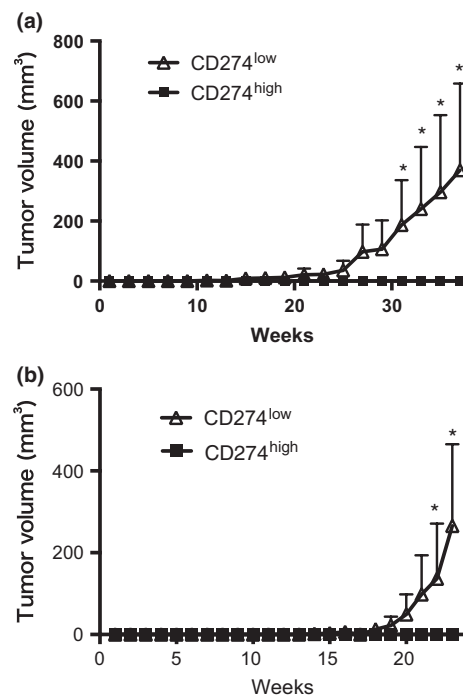
Fig. 1. Low CD274 is a candidate marker for cholangiocarcinoma cancer stem cells. Tumorigenicity of RBE (a) and HuCCT1 (b) CD274^{low} and CD274^{high} cells. The average tumor volume is shown with the SEM (a: $n = 6$, 10^4 cells, $*P < 0.05$; b: $n = 4$, 10^2 cells, $*P < 0.05$).

tion solvent systems were (i) 10 mM ammonium acetate (pH 6.5) : methanol (40:60 v/v) and (ii) methanol. The gradient was linear over 20 min from 0 to 100% solvent (ii) at a flow rate of 1.0 mL/min. All assays were performed in triplicate.

Statistical analysis

Survival curves were calculated with R statistical software according to the Kaplan–Meier method and log-rank analysis. Pearson χ^2-test was used on univariate analysis in patient demographics. In tumorigenicity assay[23], statistical analyses were performed with R statistical software according to the Mann–Whitney U-test.

Results

CD274^{low} population possesses a high tumor-initiating potential. To investigate whether CD274 is involved in tumor initiation, we sorted a human cholangiocarcinoma cell line, RBE cells based on their expression of CD274 (Fig. S1), and the CD274^{low} and CD274^{high} cells were then xenografted into immunodeficient NOD/scid/γcnull (NOG) mice. The CD274^{low} population (1×10^4 cells) formed tumors at all six (6/6) injection sites, whereas the CD274^{high} population formed tumors in one out of six (1/6) injection sites after 25 weeks of xenograft (Fig. 1a, Table 1A). We also examined the relationship between the expression level of CD274 and the tumorigenic potential using another human cholangiocarcinoma cell line, HuCCT1. The CD274^{low} population (1×10^2 cells) of HuCCT1 formed tumors at three out of four (3/4) injection sites, whereas the CD274^{high} population formed no (0/4) tumor after 23 weeks of xenograft (Fig. 1b, Table 1B). These results suggest that the CSC of cholangiocarcinoma are enriched in the CD274^{low} population.

CD274^{low} cells possess CSC-related characteristics. We then investigated the CSC-related characteristics of the CD274^{low} and CD274^{high} populations derived from RBE and HuCCT1 cells. They were sorted by anti-CD274 antibody staining and the resulting populations were assessed for ALDH activity using the Aldefluor assay system followed by FACS analysis. The RBE CD274^{low} population was 46.1% ALDH$^+$,

whereas the RBE CD274^{high} population was 10.2% ALDH$^+$ (Fig. 2a). The HuCCT1 CD274^{low} and CD274^{high} populations showed 52.4% and 38.3% ALDH$^+$, respectively. We then measured the retinal metabolism *in vitro*, because ALDH metabolizes all-trans retinals to all-trans retinoic acids. The level of retinoic acids produced by the CD274^{low} population was significantly higher than that produced by the CD274^{high} population (Fig. 2c,d) in both RBE and HuCCT1 cells. These results are compatible with those of the Aldefluor assay. The CD274^{low} and CD274^{high} populations were further assessed for ROS levels. The ROS levels were significantly lower in the CD274^{low} populations than in the CD274^{high} populations derived from both RBE and HuCCT1 cells (Fig. 2e,f). These results indicate that the CD274^{low} populations of both cholangiocarcinoma cell lines predominantly exhibit CSC-related characteristics such as high ALDH1 activity and low ROS production.

Because hematopoietic and leukemic stem cells are known to reside largely in the G0 phase, we examined the cell cycle phases of the RBE and HuCCT1-derived CD274^{low} and CD274^{high} populations. The CD274^{low} and CD274^{high} populations were stained with the DNA-binding dye propidium iodide for defining the DNA content and an anti-Ki-67 antibody for defining the G0 phase. Most of the CD274^{low} cells from both cell lines were found to be Ki-67$^-$, corresponding to the G0 phase, whereas the CD274^{high} cells showed various phases of the cell cycle (Fig. 2g,h). These results indicate that the CD274^{low} cells derived from both RBE and HuCCT1 exhibit the characteristics of dormancy, which is often detected in the CSC of other types of cancer.

The CSC are known to express embryonic transcription genes such as *Sox2*, *Nanog* and *Oct4*. Therefore, we compared the expression profiles of these genes between the CD274^{low} and CD274^{high} cells derived from RBE and HuCCT1. Real-time PCR analyses showed more elevated levels of *Oct4*, *Sox2* and *Nanog* in the CD274^{low} cells than in the CD274^{high} cells (Fig. 2i,j). Thus, the gene expression profile of the CD274^{low} cells was consistent with the characteristics of CSC.

CD274^{low} cells possess plasticity and differentiation capabilities. We then investigated the plasticity of the CD274^{low} popu-

Table 1. Tumor initiation capability. (A, B) Tumor initiation capability of the CD274^{low} and CD274^{high} cells in RBE (A) and HuCCT1 (B) cells. (C,D) Tumor initiation capability of control versus RNAi-1 and RNAi-2 cells in RBE cells

Population	No. cells injected	Weeks											
(A)													
		1	5	9	13	17	21	25	29	33	37		
CD274^{high}	10 000	0/6	0/6	0/6	0/6	1/6	1/6	1/6	1/6	2/6	2/6		
CD274^{low}	10 000	0/6	0/6	0/6	4/6	5/6	5/6	6/6	6/6	6/6	6/6		
(B)													
		1	3	5	7	9	11	13	15	17	19	21	23
CD274^{high}	100	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
CD274^{low}	100	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1/4	2/4	3/4
(C)													
		2	6	10	14	18	22	26	30	34			
Control	100 000	0/4	0/4	0/4	0/4	0/4	0/4	0/4	1/4	1/4			
RNAi-1	100 000	0/4	0/4	0/4	0/4	1/4	2/4	4/4	4/4	4/4			
(D)													
		20	24	28	32	36	40	44	48	52			
Control	10 000	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6			
RNAi-2	10 000	0/6	0/6	0/6	0/6	0/6	0/6	1/6	1/6	3/4			
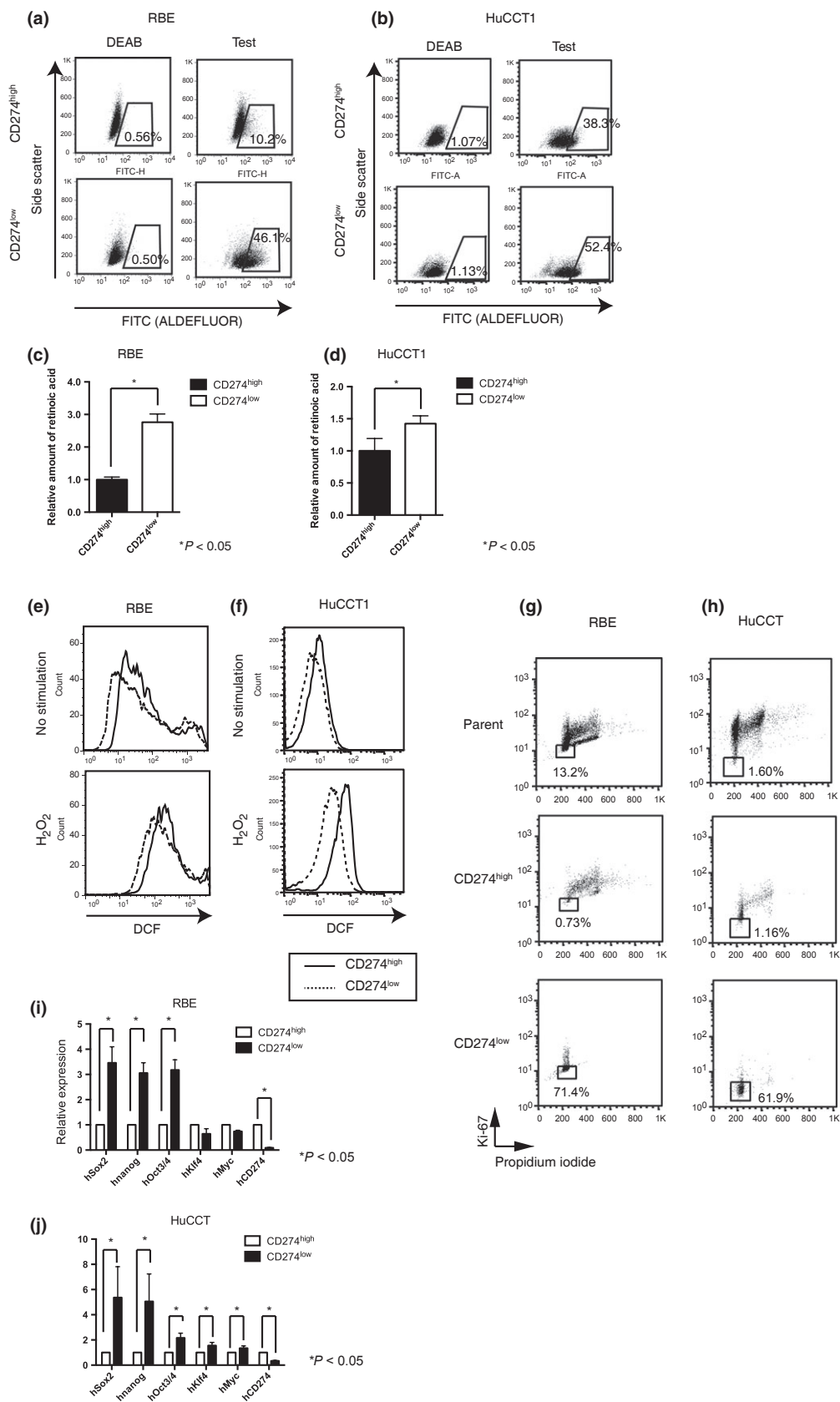
lations of RBE and HuCCT1 cells. The CD274^{low} and CD274^{high} cells were cultured and stained for CD274 periodically during *in vitro* culture. The RBE CD274^{low} cells showed

an increase of CD274 expression and shifted to a CD274^{high} state at day 3 of culture, and vice versa, the RBE CD274^{high} cells showed a decrease of CD274 expression (Fig. 3a). Simi-

Fig. 2. (On the previous page) Evaluation of cancer stem cell (CSC)-related phenotypes in CD274^{low} subpopulations. (a,b) CD274^{low} and CD274^{high} populations were sorted from the RBE and HuCCT1 cell lines as described in Supporting Information Fig. S1. Aldehyde dehydrogenase (ALDH) activity of the RBE (a) and HuCCT1 (b) subpopulations was measured using the Aldefluor assay followed by FACS analysis. A specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), was used to control for background fluorescence. (c,d) Measurement of all-trans retinal metabolism in CD274^{high} and CD274^{low} subpopulations in RBE (c) and HuCCT1 (d) cells. (e,f) Quantification of the reactive oxygen species (ROS) production in RBE (e) and HuCCT1 (f) subpopulations. Each subpopulation was incubated with or without H_2O_2, stained with 2′, 7′-Dichlorodihydrofluorescin diacetate (DCFH-DA) and subjected to FACS analysis. (g,h) Analysis of cell cycling in the RBE (g) and HuCCT1 (h) subpopulations. CD274^{low} and CD274^{high} cells were fixed with ethanol, stained with Ki-67 FITC and propidium iodide and subjected to FACS analysis. (i,j) Expression profile of stem cell-related genes in CD274^{high} and CD274^{low} cells. mRNA from CD274^{high} and CD274^{low} cells derived from the RBE (i) and HuCCT1 (j) cell lines were analyzed using real-time PCR.

lar shifts of CD274 expression were observed on the CD274^{low} and CD274^{high} populations of HuCCT1 cells, although the HuCCT1 CD274^{low} cells started to shift to a CD274^{high} state within 24 h of culture (Fig. 3b). These results suggest that the CD274^{low} cells have plasticity *in vitro*. Next, we investigated the characteristics associated with the *in vivo* plasticity of RBE CD274^{low} cells. Tumors formed in NOG mice engrafted with the RBE CD274^{low} cells were analyzed for the expression of CD274 and Ki-67 using immunohistochemistry. CD274 was

expressed predominantly at the periphery of tumor nodules, as was Ki-67 (Fig. 3c). These results suggest that the CD274^{low} cells possess plasticity and are in a dormant state *in vivo*.

CD274 regulates CSC-related phenotypes. To determine whether CD274 directly regulates CSC-related phenotypes, we established CD274-knockdown RBE cells by introducing retroviral vectors expressing CD274 shRNA (Fig. 4a). The cell proliferation curves were not significantly different between CD274-knockdown cells and the control (Fig. 4b)
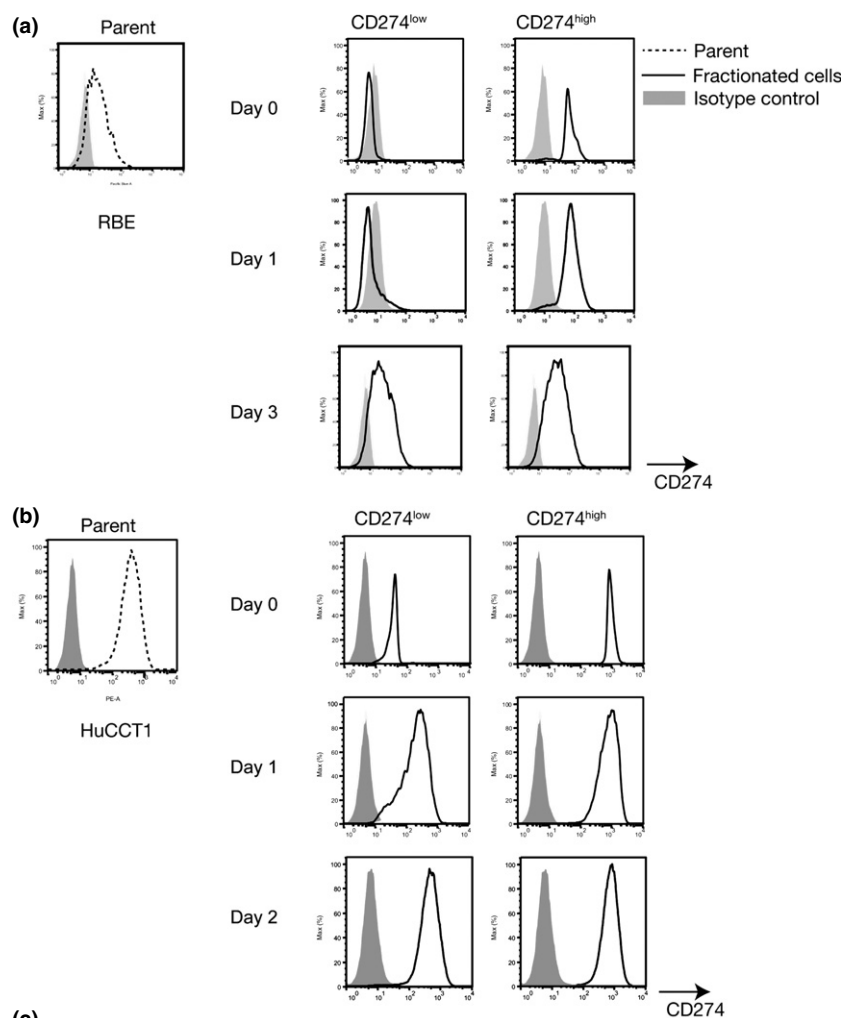
Fig. 3. Interconversion of the CD274^{high} and CD274^{low} states *in vitro*. RBE (a) or HuCCT1 (b) cells were sorted using FACSAria and cultured under normal conditions. After incubation for the indicated times, the cells were stained with an anti-CD274 antibody and analyzed using flow cytometry. (c) Immunohistochemistry of tumor cells 38 weeks after the engraftment of CD274^{low} cells into immunodeficient NOD/scid/γcnull mice. Bar, 100 μm. The tumor was fixed and stained with anti-CD274 (brown) and anti-Ki-67 (red) antibodies.
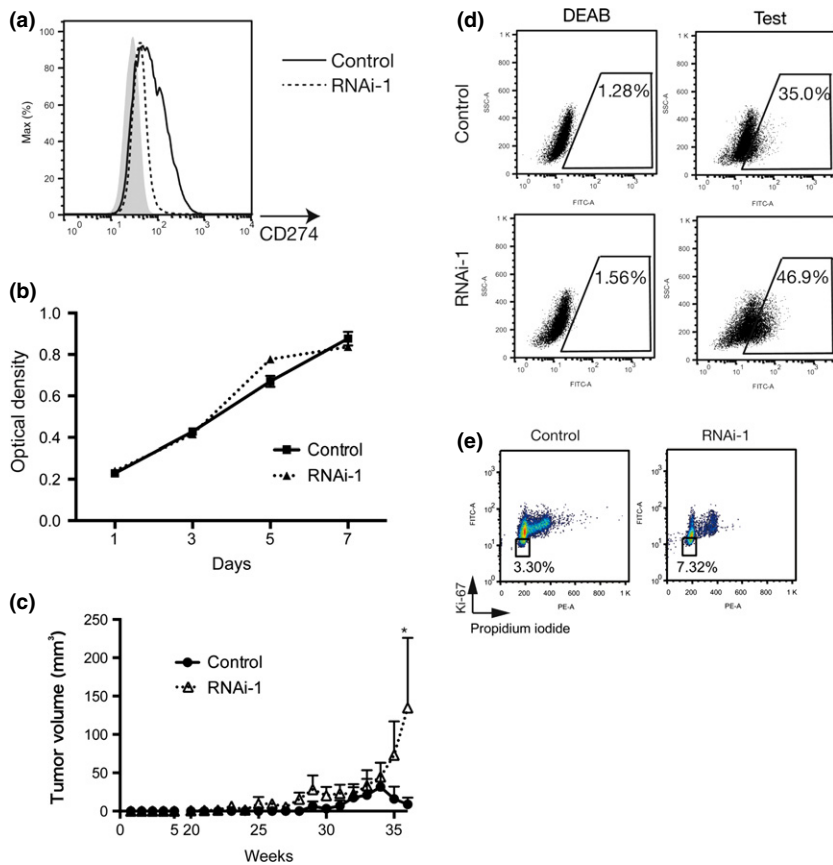
Fig. 4. CD274 directly regulates cancer stem cell (CSC) phenotypes in cholangiocarcinoma cell lines. (a) Flow-cytometric analysis of control and CD274-knockdown RBE cells (RNAi-1). (b) Proliferation of control and CD274-knockdown RBE cells assessed using MTT assay. (c) Tumorigenicity of control and CD274-knockdown RBE cells. $n = 4$, 10^5 cells, $*P < 0.05$. (d) aldehyde dehydrogenase (ALDH) activity in each of the cell lines measured using the Aldefluor assay. Diethylaminobenzaldehyde (DEAB) was used to control for background fluorescence. (e) Analysis of cell cycling in the CD274-knockdown and control cells. Following puromycin selection for 2 days, cells were fixed with ethanol, stained with Ki-67 FITC and propidium iodide and subjected to FACS analysis.

after the puromycin selection for 14 days. The CD274-knockdown cells displayed a higher tumorigenic capability in NOG mice than the control cells (Fig. 4c, Table 1C). The ALDH activity was also increased in the CD274-knockdown cells (Fig. 4d). In cell cycle analysis, CD274-knockdown cells exhibit relatively in the G0/G1 phase compared with control cells after puromycin selection for 2 days (Fig. 4e). We confirmed these phenotypes using another target sequence (RNAi-2) and obtained similar results (Table 1D, Fig. S2). Taken together, these results indicate that CD274 functions to suppress CSC-related phenotypes in the RBE cholangiocarcinoma cells.

Histological profile of clinical samples. Last, we addressed the relationship between CD274 expression in clinical specimens and the prognosis of patients with cholangiocarcinoma. Tumor tissue specimens were completely resected from 91 patients with cholangiocarcinoma, as assessed both surgically and histologically. Anti-CD274 antibody staining showed high CD274 expression in the normal extrahepatic ductal areas (Fig. 5a). In cancerous areas, CD274 was homogeneously expressed in the ductal regions, but ranged from weak to strong (Fig. 5b,c) among the samples. We then divided the samples into two groups, CD274$^{-/+}$ and CD274^{++}, according to the expression level of CD274 in their cancerous areas, and compared the overall survival of patients using the Kaplan–Meier methods. The CD274$^{-/+}$ group showed a significantly shorter median survival time than the CD274$^{+/+}$ group ($P = 0.0157$; Fig. 5d). There was no significant correlation between the expression level of CD274 and clinical demographics including age, sex or pathological stage of the cancer (Table S2). These results

indicate that a lower expression of CD274 is correlated with a poorer prognosis in cholangiocarcinoma.

Discussion

In the present study using the two human cholangiocarcinoma cell lines, RBE and HuCCT1, we first demonstrated that the CD274^{low} cells derived from the two cell lines possess higher tumorigenecity in NOG mice compared with the CD274^{high} cells. Both RBE and HuCCT1 hardly expressed PD-1 (Fig. 3s), which is known for the receptor of CD274, and NOG mice have multiple immunological dysfunction including T cells[24] indicating a possible involvement of CD274 in regulation of CSC characteristics of human cholangiocarcinoma without the involvement of CD274-PD-1 interaction.

It is generally known that CSC in leukemia cycle through a state of deep long-term quiescence or dormancy.[8,25] The existence of some slow-growing cancer cell populations has also been shown in solid tumors such as breast and liver cancers.[7,26] In the present study, RBE and HuCCT1-derived CD274^{low} cells resided primarily in the G0 dormant phase and the amount of retinoic acid was significantly increased in CD274^{low} cells. Because it is well known that retinoic acid causes G0 cell cycle arrest,[27] retinoic acid is involved in the regulation of cell cycle arrest in CD274^{low} cells. In NOG mice transplanted with RBE-derived CD274^{low} cells, the proliferating cells detected using Ki-67 staining colocalized mainly with CD274^{high} cells in tumor tissues, suggesting that the CD274^{low} cells are also relatively dormant *in vivo* as well as *in vitro*. The expression of CD274 in breast cancer is reportedly associated with highly proliferative Ki-67-expressing tumor cells,[17] which is compatible with our
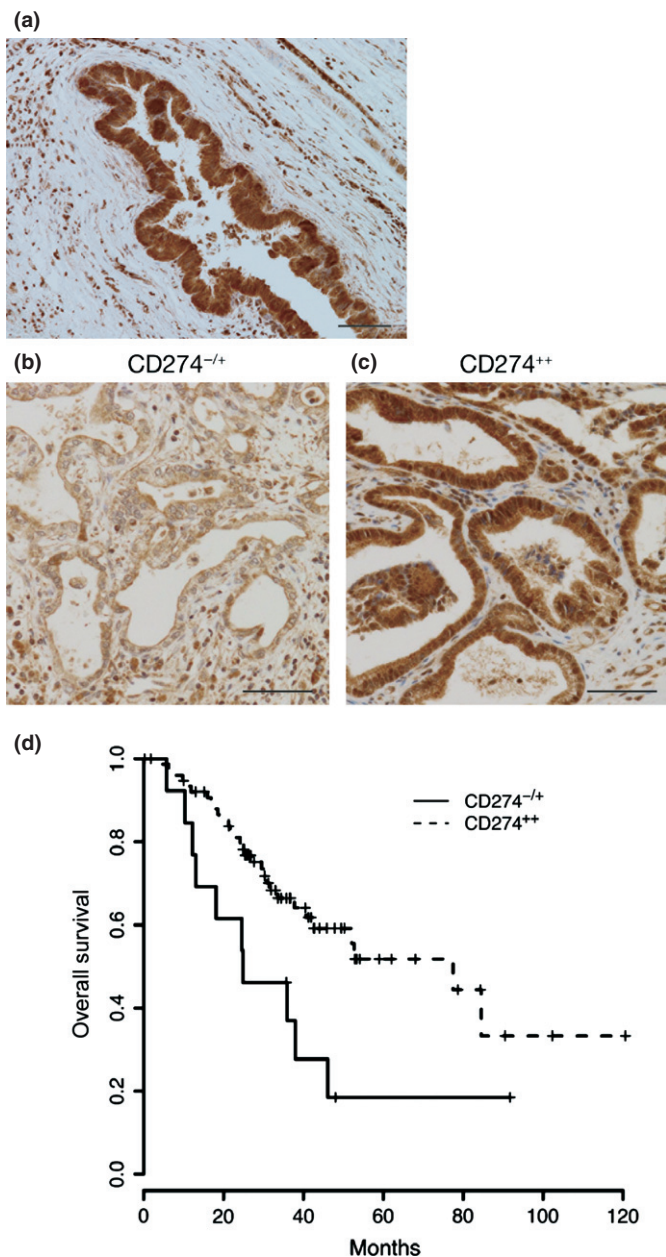
Fig. 5. CD274 expression in cholangiocarcinoma specimens and prognosis of cholangiocarcinoma patients. (a) Normal bile duct staining with an anti-CD274 antibody. Bar, 100 μm. (b,c) Representative results of the anti-CD274 staining of clinical specimens obtained from 91 patients by surgical resection. CD274 immunohistochemistry was scored as described in Materials and Methods. Bar, 100 μm. (d) Kaplan–Meier analysis for the disease-specific survival rate of the two groups.

observation. In addition, *in vitro* cultures of the cholangiocarcinoma cell lines showed that CD274^{low} cells were capable of producing CD274^{high} cells, and vice versa, CD274^{high} cells were also capable of producing CD274^{low} cells, although the transition from CD274^{high} to CD274^{low} was considerably slower (Fig. 3b). It is generally known that some tumors possess the capability of reversible transition between tumorigenic and non-tumorigenic states.[28] Collectively, these findings suggest that there is a hierarchy in the expression of CD274 on cholangiocarcinoma cells and that CD274^{low} cells tend to be dormant, similar to other CSC, whereas CD274^{high} cells possess a higher proliferative potential than CD274^{low} cells. Furthermore, because high ALDH and low

ROS activities are known to be other characteristics of CSC in several types of cancer,[5,6] we compared these activities between the CD274^{low} and CD274^{high} populations of RBE and HuCCT1 cells. The CD274^{low} populations derived from both cell lines showed higher ALDH and lower ROS activities than the CD274^{high} populations, indicating that the CD274^{low} cells of cholangiocarcinoma carry the characteristics of CSC.

The CSC of various cancers are known to have similar characteristics to induced pluripotent stem (iPS)-embryonic stem (ES) cells regarding the expression of genes such as *Nanog*, *Sox2* and *Oct3/4*. *Nanog* has a role in inducing CSC pluripotency in various cancers and the overexpression of *Nanog* leads to elevated tumorigenicity, while its inhibition reduces tumorigenecity in prostate, breast and colorectal cancers.[29–31] It is also reported that Sox2 increases the expression of CSC markers and the *in vivo* tumor-initiating capacity.[32] We also found that the mRNA for *Nanog*, *Sox2* and *Oct3/4* were increased in the CD274^{low} populations derived from both RBE and HuCCT1 cell lines, indicating that the CD274^{low} population has pluripotent stem cell-like characteristics that correlate with iPS/ES cell-related gene expression. These gene expression profiles also suggest that the CD274^{low} cells act as CSC.

The relationship of CD274 expression to tumor aggressiveness, clinicopathological features and overall survival is well known in several human malignancies, such as ovarian, esophageal, pancreatic and non-small-cell lung cancers, indicating that high CD274 expression on these cancer cells in patients induces an immunosuppressive response resulting in tumor progression.[33–37] In contrast with these reports, our immunohistochemical analysis of CD274 expression in cholangiocarcinoma specimens revealed that a low expression of CD274 correlated well with a poor prognosis for the patients. To resolve these incompatible observations regarding the effects of CD274 on cancer progression, we independently reviewed non-small-cell lung cancer specimens for the expression of CD274 and disease-free survival time and obtained the result that high expression of CD274 in non-small-cell lung cancer specimens tended to shorten the patients' disease-free survival time (data not shown), which confirmed the observations reported previously.[33,35] Thus, we become convinced that CD274 has a novel function to suppress malignant phenotypes of cholangiocarcinoma, which may overcome its immunosuppressive effect.

Many surface molecules including CD133 were identified as CSC markers in several types of cancer,[11,12] although one previous report suggested that in colorectal cancer cells, CD133 was not regarded as a CSC marker.[38] We examined whether this marker also functions as CSC markers in cholangiocarcinoma. In HuCCT1 cells, the CD133^{high} population possessed a higher tumorigenicity compared with the CD133^{low} population (Fig. S4A,B). In contrast, in RBE cells, the CD133^{low} population showed a higher tumorigenicity compared with the CD133^{high} population (Fig. S4C,D). Taken together, CD133 is not a suitable markers for CSC of cholangiocarcinoma.

In the present study, we revealed that the CD274^{low} cholangiocarcinoma cells have several CSC-related phenotypes. Furthermore, CD274 knockdown in RBE cells endows them with the CSC-related phenotypes. Based on our present study, we propose that in cholangiocarcinoma, CD274 possesses a suppressive function against the induction or maintenance of CSC.

Acknowledgments

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Flow cytometry histograms showing CD274 expression in cholangiocarcinoma cell lines.

Fig. S2. Characterization of CD274-knockdown RBE cells using the RNAi-2 target sequence.

Fig. S3. Expression of PD-1 in cholangiocarcinoma cell lines.

Fig. S4. Evaluation of the tumorigenicity in CD133-positive subpopulations in cholangiocarcinoma cell lines.

Table. S1. Primers used in the present study.

Table S2. Patient demographics.