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Role of histone deacetylase 1 in distant metastasis of pancreatic ductal cancer

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Current therapies for pancreatic ductal cancer (PDAC) do not sufficiently control distant metastasis. Thus, new therapeutic targets are urgently needed. Numerous studies have suggested that the epithelial-mesenchymal transition (EMT) is pivotal for metastasis of carcinomas. The fact that the EMT is reversible suggests the possibility that it is induced by an epigenetic mechanism. In this study, we aimed to investigate the role of histone deacetylase 1 (HDAC1), which is an epigenetic mechanism on distant metastasis of PDAC. We investigated the HDAC1 expression in 103 resected PDAC specimens obtained from patients who were treated with/without preoperative therapy using immunohistochemistry. To validate the findings in the clinical samples, we evaluated the HDAC1 activity, the EMT-associated genes and the migration/invasion ability in vitro, and performed an HDAC1 inhibitor assay. The high expression of HDAC1 in clinical samples was significantly associated with poor progression-free survival, especially distant metastasis-free survival. In vitro, HDAC1 inhibitors decreased the invasion ability and reversed the EMT change; the only factor to show a concomitant decrease was the expression of SNAIL. We confirmed that the HDAC1 expression was associated with the SNAIL expression in clinical samples. Moreover, the resistant cells and parental cells did not show any significant differences in the expression of HDAC1; this was consistent with the finding that preoperative therapy did not alter the HDAC1 expression in clinical samples. The targeting of HDAC1, which could suppress metastasis by inhibiting the EMT, is a promising treatment option for PDAC.

KEYWORDS

distant metastasis, epithelial-mesenchymal transition, histone deacetylase 1, pancreatic ductal cancer, SNAIL

The use of clinical samples was approved by the Human Ethics Review Committee of the Graduate School of Medicine, Osaka University (17277).

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a fatal neoplasm with poor prognosis.[1,2] Despite developments in detection and management, only approximately 8% of PDAC patients survive for 5 years after diagnosis.[3-5] Surgery is the only curative treatment for PDAC; however, only 20% of cases are considered resectable at the time of detection.[6] Furthermore, 20% of resectable PDAC develop local recurrence and 70% develop distant recurrence after surgery.[7] With regard to recurrence, PDAC cells spread to both the surrounding tissue and distant organs in the early phase after carcinogenesis; thus, multidisciplinary therapy (surgery plus adjuvant chemotherapy) is highly recommended for all PDAC patients to manage the tumor cells that possess invasion/migration ability. Multidisciplinary therapy improves the overall survival of patients with resectable PDAC; nevertheless, the 5-year survival rate of 44.1% is not satisfactory. Taken together, approximately 95% of patients with PDAC show distant metastasis during their clinical course, and the present therapies do not sufficiently control cancer cells that possess invasion/migration ability.[8] Thus, new therapeutic targets are urgently needed to overcome metastasis in PDAC.

Epithelial-mesenchymal transition (EMT) is the differentiation process through which epithelial cells are converted into mesenchymal-like cells. Recently, numerous differences have been observed between epithelial cells and mesenchymal cells, and it was discovered that the EMT causes cells to lose their junction and apical-basal polarity, reorganize their cytoskeleton, undergo changes in the signaling programs that define their shape, and reprogram their gene expression. As a consequence of these changes, the cells show increased motility and invasion ability.[9] Numerous studies have suggested that the EMT is pivotal for the invasion and metastasis of carcinoma.[10-12] The changes in the EMT were observed in cancer cells and were induced in vitro. In the experiments, the changes in the EMT-induced cells were not permanent, which suggested the possibility that the EMT is induced by a certain epigenetic mechanism and not by a genetic change.

Histones have numerous modifications, including (but not limited to) acetylation, methylation, phosphorylation, poly-ADP ribosylation, ubiquitination, sumoylation and carbonylation.[13] With regard to histone modifications, lysine acetylation depends on the antagonistic activity of 2 enzyme classes: histone acetylases (HAT) and histone deacetylases (HDAC).[14] We previously revealed that HDAC1, one of the enzymes controlling epigenetic mechanisms, was associated with the EMT in biliary tract cancer.[15] However, there is no critical consensus on the association between the EMT and HDAC1, or in the role of HDAC1 in the multidisciplinary treatment of PDAC.

Therefore, we aimed to investigate the role of HDAC1 in distant metastasis of PDAC. We found that the expression of HDAC1 was positively associated with distant metastasis in patients with resectable PDAC. These findings were validated using PDAC cells. Furthermore, we investigated the effect HDAC inhibitors on metastasis to establish a new approach for treating PDAC with metastasis.

2 | MATERIALS AND METHODS

2.1 | Cell lines, culture and drugs

This study used 4 human PDAC cell lines (BxPC3, MiaPaCa2, Panc1 and PSN1). BxPC3 and MiaPaCa2 were obtained from the Japan Cancer Resource Bank (Tokyo, Japan), Panc1 was obtained from the ATCC and PSN1 was obtained from the European Collection of Authenticated Cell Culture. The cells were grown in DMEM supplemented with 10% FBS, and 100 units/mL penicillin at 37°C in a humidified incubator with 5% CO_2. We used stable GEM-resistant cell clones established from MiaPaCa2 cells by long-term exposure to GEM, which was purchased from Eli Lilly Pharmaceuticals (Indianapolis, Indiana, USA) and named them MiaPaCa2-GR cells, as reported previously.[16] We also used radio-resistant cell clones established from Panc1 and MiaPaCa2 cells by fractionated irradiation and named them Panc1-RR and MiaPaCa2-RR cells, respectively, as reported previously.[17] In the experiment with the histone deacetylase inhibitor, Vorinostat (Selleckchem, Houston, Texas, USA) and 4-(dimethylamino)-*N*-[6-(hydroxyamino)-6-oxohexyl]-benzamide (DHOB) (Santa Cruz Biotechnology, Dallas, TX, USA), PSN1 and MiaPaCa2 were treated with/without 500 nmol/L Vorinostat and with/without 5 μmol/L DHOB for 72 hours after ascertaining that the concentration had no significant effect on the viability (Supplementary Figure S1).

2.2 | The downregulation of SNAIL gene

For siRNA suppression, we used a Silencer Select kit (Ambion, Austin, Texas, USA) with double-stranded RNA duplexes that targeted human SNAIL and negative control siRNA (NC), as previously described.[18] PSN was transfected with the siRNA in lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol.

2.3 | Clinical samples

A total of 103 quality-verified PDAC samples were obtained from patients (mean age, 67.1 ± 9.4 years; male-to-female ratio, 64:39) who underwent histologically curative pancreatic resection (R0) from March 2007 to September 2013 at Osaka University Hospital. All patients were clearly diagnosed with PDAC based on clinicopathological findings, and the median observation time was 28.3 (3.8-89.2) months. The mean tumor size was 23.0 ± 13.2. The clinicopathological features of the patients are shown in Supplementary Table S1. Resected PDAC specimens were immediately fixed in 10% formalin for 48 hours. Specimens were then embedded in paraffin and sectioned into 3.5-μm slices for further evaluations, as described previously.[19] A proportion of the slides were routinely stained with H&E for pathological evaluation by a certified pathologists in our institution. The remaining slides were examined by immunohistochemistry (described below). The use of resected samples was approved by the

Human Ethics Review Committee of the Graduate School of Medicine, Osaka University (17277), and informed consent was obtained from each of the patients.

2.4 | Preoperative therapy (chemoradiotherapy)

During these observation periods, 2 sequential clinical studies were recommended for PDAC patients who were eligible, as previously reported.[20] In the first trial, patients were treated with GEM (800 mg/m^2) and 40 Gy radiation; patients with both advanced and resectable PDAC were recommended to enroll in the study.[21] Those in the subsequent clinical trial were treated with GEM (600-1000 mg/m^2), S-1 (40-80 mg/m^2) and 50.4 Gy radiation; all patients with resectable PDAC were recommended to enroll in the study.[22] All of these patients provided their written informed consent for participation. The patients were hospitalized for 1-2 months when receiving chemoradiotherapy to observe the safety of the therapy.

2.5 | Immunohistochemistry

Immunohistochemical studies of HDAC1 and SNAIL were performed on 103 surgical specimens of PDAC, as described previously.[18,23] Briefly, formalin-fixed, paraffin-embedded tissues were deparaffinized, hydrated and incubated overnight at 4°C with specific, rabbit polyclonal, anti-HDAC1 antibodies (1: 4000 dilution; Abcam, Cambridge, MA, USA) and anti-SNAIL antibodies (1:1000 dilution; Abcam). Bound antibodies were detected with biotin-conjugated secondary antibodies and diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) as a substrate. All sections were counterstained with hematoxylin. We evaluated the expression at invasion fronts in PDAC specimens.

2.6 | Western blotting

Western blotting was performed as described previously.[24] Briefly, total protein was extracted from PDAC cell lines in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Rockford, IL, USA) and nuclear proteins were extracted with the Nuclear Extraction Kit according to the manufacturers' protocol. Aliquots of total protein (12 μg) were electrophoresed on sodium dodecyl sulfate polyacrylamide gels, 10% Tris-HCL gels (Bio-Rad Laboratories, Hercules, CA, USA). The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) and incubated with primary antibodies for 1 hour. Proteins were detected with anti-HDAC1 antibody (1:200 dilution; Santa Cruz Biotechnology), anti-SNAIL antibody (1:2000 dilution; Abcam), anti-ZEB1 antibody (1:1000 dilution; Santa Cruz Biotechnology), anti-Cytokeratin 19 antibody (1:200 dilution; Santa Cruz Biotechnology), anti-Histone H3 (1:2000; Cell Signaling Technology, Danvers, MA, USA) and anti–β-actin antibody (diluted 1:4000; Sigma, Tokyo, Japan). The expression relative to actin expression was depicted as a column and measured with ImageJ software (rsb.info.nih.gov/ij).

2.7 | Quantitative RT-PCR

The quantitative RT-PCR (qRT-PCR) was performed as previously described.[15] Briefly, total RNA was isolated from cell lines with TRIzol Reagent (Invitrogen). For the mRNA evaluations, complementary DNA was synthesized with the Reverse Transcription System (Promega, Tokyo, Japan). Then, a qRT-PCR was performed with specifically-designed oligonucleotide primers (Supplementary Table S2) and a LightCycler 480 Real-Time PCR system (Roche Diagnostics, Manheim, Germany). The amplification products were quantified with the LightCycler-DNA master SYBR Green I Kit (Roche Diagnostics). The target gene expression levels were normalized to the β-actin expression level, which served as an endogenous control.

2.8 | Immunocytochemical staining

Immunocytochemistry (ICC) was performed as described previously.[15] Briefly, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, permeabilized with 0.1% Triton X-100, and blocked with 1% BSA for 10 minutes at room temperature. Subsequently, the cells were stained with the following antibodies: anti-Cytokeratin 19 antibody (1:50 dilution; Santa Cruz Biotechnology) and anti-HDAC1 antibodies (1:1000 dilution; Abcam). After counterstaining with Hoechst 33342, Trihydrochloride and Trihydrate (Invitrogen) to visualize the nuclei, the slides were analyzed with a confocal fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan).

2.9 | Growth inhibition assays and the determination of cell viability with GEM therapy

Growth inhibition was assessed using the MTT assay (Sigma-Aldrich Co, St. Louis, MO, USA) as described previously.[25] Each cell line was seeded onto a 96-well plate (5×10^3 cells/well) and incubated for 24 hours. Subsequently, the cells were exposed to GEM, Vorinostat or DHOB (at several concentrations) for 72 hours. Cell viability was then evaluated by absorbance using MTT solution. The results were expressed as the percentage of absorbance relative to that of untreated controls.

2.10 | The histone deacetylases activity assay

Nuclear proteins were extracted with a Nuclear Extract Kit (Abcam). The HDAC1 activity was subsequently measured with an HDAC Activity Assay Kit (Epigentek, Farmingdale, NY, USA). All procedures were conducted according to the manufacturer's recommendations.

2.11 | Invasion assays

The invasion assay was performed with invasion chambers loaded with Matrigel according to the manufacturer's instructions (Biocoat Matrigel Invasion Chamber; Collaborative Biomedical Products; Becton Dickinson, Franklin Lakes, NJ, USA), as described previously.[26]

Briefly, 5×10^4 cells were overlaid onto the Matrigel matrix on a membrane with 8-mm-diameter pores with or without Vorinostat or DHOB. After 48 hours, the cells that had invaded the undersurface of the membrane were fixed with methanol and stained with thiazine and eosinate. Four microscopic fields were randomly selected for cell counting.

2.12 | Statistical analysis

Student's *t* test or Fisher's exact test were used to analyze the categorical data, and the Mann-Whitney *U* test was used to analyze the nonparametric data. A Kaplan-Meier analysis and log-rank test were performed to construct survival curves and to evaluate differences in survival. The prognostic value of each clinicopathological characteristic was first determined using a univariate Cox regression analysis. The parameters that were significantly associated with survival in the univariate analysis were included in a multivariate analysis to identify significant clinicopathological factors. *P*-values of <0.05 were considered to indicate statistical significance. All the statistical analyses were performed using the JMP 13.0 software program (SAS Institute, Cary, NC, USA).

3 | RESULTS

3.1 | High levels of histone deacetylase 1 were associated with earlier relapse

We investigated HDAC1 expression in resected specimens. All the PDAC tissue samples showed the expression of HDAC1 somewhere; nevertheless, there was wide variation in the number of cells that were HDAC1-positive. We divided cases into 2 groups according to the number of cells that were HDAC1-positive: the HDAC1-high group ($HDAC1^{high}$; n = 52) and the HDAC1-low group ($HDAC1^{low}$; n = 51, Figure 1A). The $HDAC1^{high}$ group showed a significantly poorer prognosis, in terms of the progression-free survival time (PFS) in comparison with the $HDAC1^{low}$ group (Figure 1B). With the exception of the pathological differentiation of tumors, there were no significant differences in the clinicopathological features (including the tumor stage and the presence/absence of preoperative therapy) of the 2 groups (Supplementary Table S3).

The univariate analysis of factors associated with PFS (Table 1) revealed several clinicopathological features that were significantly associated with PFS: the serum CEA level ($P < 0.0001$), the presence of jaundice ($P = 0.0185$), completion of adjuvant therapy ($P = 0.0042$), Union for International Cancer Control 7th edition (UICC) pathological stage ($P = 0.0364$), UICC pathological T stage ($P = 0.0305$), tumor size ($P = 0.0296$), the presence of lymph node metastasis ($P = 0.0138$) and the expression of HDAC1 ($P = 0.0194$). The multivariate analysis of these risk factors revealed that the HDAC1 expression (as determined by IHC) was an independent prognostic factor ($P = 0.0207$), as were the CEA level ($P < 0.0001$), the presence of jaundice ($P = 0.0091$) and the completion of adjuvant therapy ($P = 0.0146$).

To determine the type of relapse that was most related to the expression of HDAC1, distant metastasis-free survival (DMFS) and local progression-free survival (LPFS) were evaluated. The DMFS in the $HDAC1^{high}$ group was significantly shorter than that in the $HDAC1^{low}$ group ($P = 0.0123$), whereas there was no significant difference in the LPFS of the 2 groups ($P = 0.1348$, Figure 1C). Taken together, these findings suggest that a large number of HDAC1-positive PDAC cells indicated the presence of occult distant metastasis. Thus, it was suggested that HDAC1 induced the migration/invasion ability in PDAC cells. Furthermore, chemotherapy and radiotherapy did not seem to affect the HDAC1 expression because preoperative therapy did not change the ratio of the HDAC1 expression.

To understand the influence of HDAC expression on the sensitivity for chemotherapy in clinical situation, the presence of preoperative therapy and the survival time after recurrence were investigated. The presence of preoperative therapy did not change the prognosis of distant metastasis in either the $HDAC1^{high}$ or the $HDAC1^{low}$ group ($P = 0.2090$, $P = 0.2108$, Supplementary Figure S2a,b). Furthermore, the survival time after recurrence, which is mainly influenced by the chemosensitivity of PDAC, was not associated with the expression of HDAC1 ($P = 0.4280$, Supplementary Figure S3a), indicating that the expression of HDAC1 was associated with metastasis in PDAC regardless of whether chemotherapy or radiotherapy was administered.

3.2 | Histone deacetylases activity inhibitor suppressed the epithelial-mesenchymal transition in pancreatic ductal cancer cells by targeting SNAIL

As it was indicated that HDAC1 regulated the migration/invasion ability of PDAC cells, we validated these findings in vitro. Four types of PDAC cell lines showed various HDAC1 activities, and the invasion ability of each cell line was correlated with the HDAC1 activity, as expected (Figure 2A-C). To detect the target gene that should be involved in this mechanism, we evaluated EMT-associated genes. PDAC cells with high HDAC1 activity showed the lower expression of an epithelial gene (CK-19); in contrast, the mesenchymal gene's expression (Vimentin) was increased (Figure 2D-F). Transcription factors that are reported to be associated with the EMT were examined, and both SNAIL and ZEB1 were found to be increased in PDAC cells with high HDAC1 activity (Figure 2D). To ascertain the role of HDAC1 activity in those cells, HDAC1 inhibitor assay was performed. We evaluated the changes in the invasiveness and in the EMT-related genes. It was determined that HDAC1 inhibitors, both DHOB (HDAC1-selective inhibitor) and Vorinostat (pan-HDAC inhibitor), decreased invasion ability (Supplementary Figure S4a and Figure 3B) and reversed the change in EMT (Supplementary Figure S4b and Figure 3), and only the expression of SNAIL was concomitantly decreased with the change of EMT. As there is no significant difference between the effects of both inhibitors, we assumed that the degradation of HDAC1 activity mainly impacts on inhibiting EMT by Vorinostat. As Vorinostat is already clinically available, we evaluated experiments with

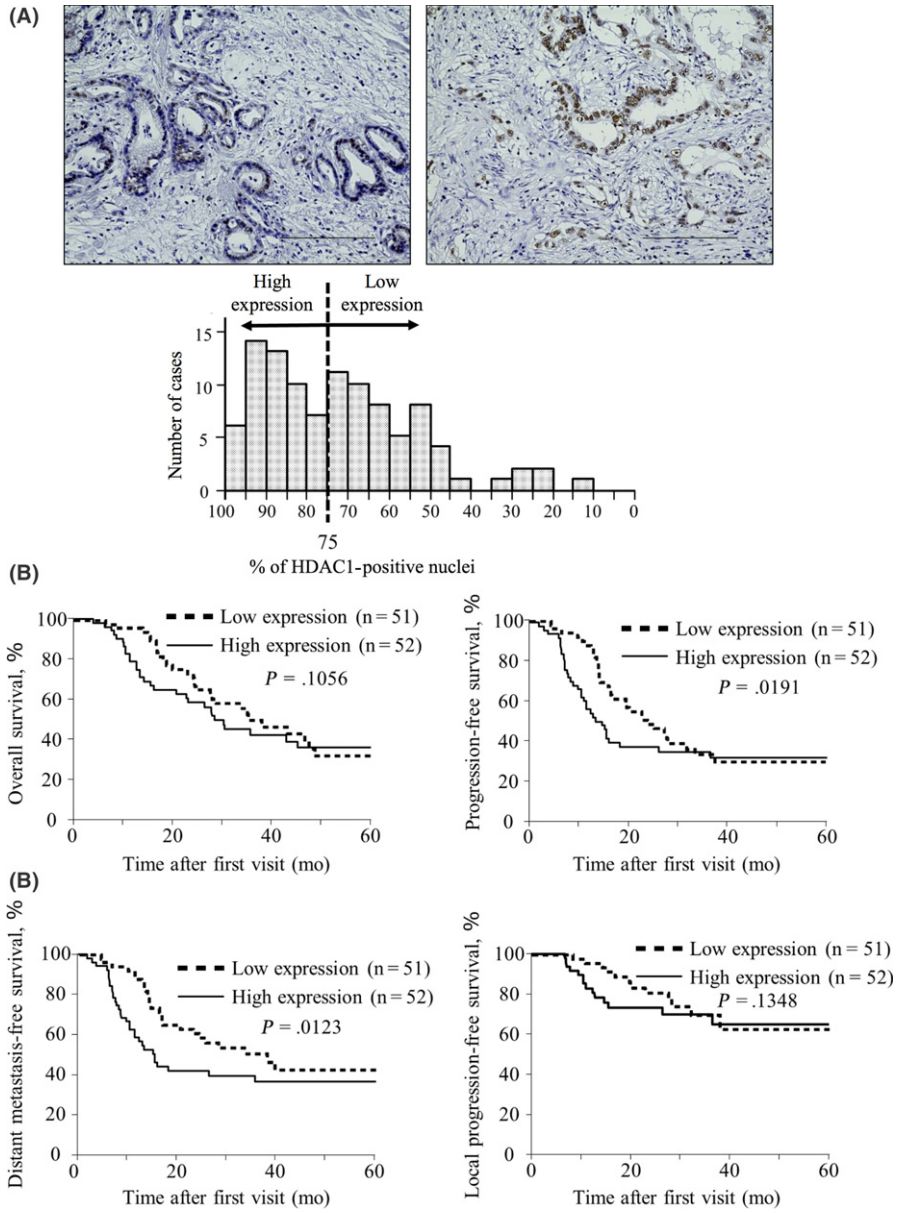


FIGURE 1 The histone deacetylase 1 (HDAC1) expression in pancreatic ductal adenocarcinoma (PDAC) and the prognosis of patients. A, The panels show representative samples with low (left) and high (right) levels of histone deacetylase 1 (HDAC1), as determined by immunohistochemical staining. Scale bar: 200 μm. The distribution of the percentage of HDAC1-positive nuclei is depicted in the lower panel. The percentage of HDAC1-positive nuclei was calculated by dividing the number of positive nuclei by the total number of nuclei of PDAC cells in the same range. The cutoff value (ratio of positive nuclei) that was used to categorize the cases into the $HDAC1^{high}$ (n = 51) and $HDAC1^{low}$ (n = 52) groups was 75%; this value was used because it was the median percentage of all cases. B, The overall survival (OS, left) and progression-free survival (PFS, right) curves after the first visit of 103 patients with PDAC. The patients were divided into groups according to the HDAC1 expression. Median OS, $HDAC1^{high}$ and $HDAC1^{low}$: 28.4 and 37.4 mo, respectively; $P = 0.1056$. Median PFS, $HDAC1^{high}$ and $HDAC1^{low}$: 14.6 and 23.8 mo, respectively; $P = 0.0191$. C, The distant metastasis-free survival (DMFS, left) and local progression-free survival (LPFS, right) curves after the first visit. The patients were divided into groups according to the HDAC1 expression. The median DMFS, $HDAC1^{high}$ and $HDAC1^{low}$: 15.4 and 37.5 mo, respectively; $P = 0.0123$. The median LPFS, $HDAC1^{high}$ and $HDAC1^{low}$: did not reach 50% (NR); $P = 0.1348$

Vorinostat to plan the next clinical study. To examine the role of SNAIL expression on EMT in PDAC, we investigated the alternation of EMT-associated genes in PSN1 with the downregulation of *SNAIL* gene. The downregulation of *SNAIL* gene reversed the change of EMT in PSN1 (Supplementary Figure 4A). These findings suggest that SNAIL is the dominant target protein in the mechanism that induces the invasive ability of PDAC cells through HDAC1 activation. In contrast, preoperative therapy did not alter the HDAC1 expression in clinical samples, and we validated this using chemotherapy/radiotherapy-resistant PDAC cells, which were

TABLE 1 The univariate and multivariate analyses of factors associated with progression-free survival

Variables	n	MST (months)	Univariate analysis *P*-value	Multivariate analysis HR	95% CI	*P*-value
Age (≤65:>65 y)	38:65	22.6:15.8	0.3591			
Gender (male:female)	64:39	20.0:16.6	0.8812			
CEA (>5:≤5 ng/mL)	20:83	9.1:26.1	**<0.0001**	6.28	3.17-12.32	**<0.0001**
CA19-9 (>37:≤37 U/mL)	71:32	15.8:31.7	0.0517			
Jaundice (present:absent)	28:75	15.8:23.8	**0.0185**	2.14	1.20-3.69	**0.0091**
Preoperative therapy (yes:no)	64:39	16.3:22.7	0.9442			
Adjuvant therapy (yes:no)	82:21	16.6:25.2	0.9008			
Completion of adjuvant therapy (yes:no)	65:38	22.6:12.2	**0.0042**	0.51	0.31-0.87	**0.0146**
Location (head:body or tail)	66:37	16.6:23.8	0.5283			
UICC pStage (III or IV:I or II)	3:100	8.2:19.3	**0.0364**	4.12	0.93-12.88	0.0598
UICC pT (I:II or III or IV)	19:84	33.3:15.8	0.0305			
Tumor size (>20:≤20 mm)	51:52	27.2:14.6	**0.0296**	1.01	0.58-1.76	0.9756
UICC pN (1:0)	35:68	12.8:22.6	0.0138			
Differentiation (tub1:tub2 or por or muc)	4:99	N/A:16.6	0.1593			
Surgery type (PD or TP or PR:DP)	68:35	16.3:25.2	0.2516			
Operation time (>480:≤480 min)	51:52	18.2:22.6	0.9972			
Blood loss (>700:≤700 mL)	49:54	16.0:23.8	0.1623			
Morbidity (present:absent)	55:48	16.3:25.2	0.7213			
HDAC IHC expression (high:low)	52:51	14.6:23.8	**0.0194**	1.83	1.10-3.08	**0.0207**

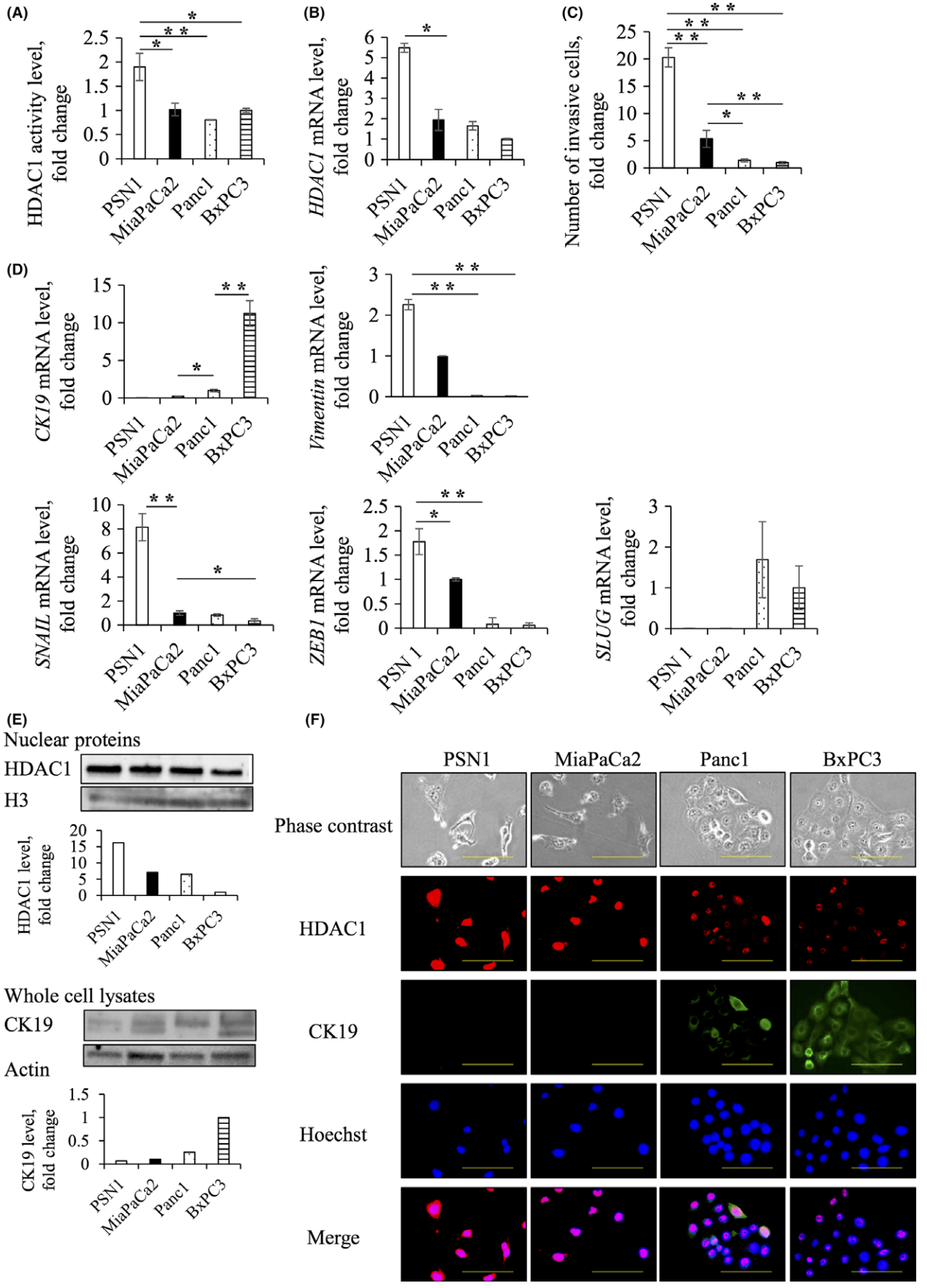
DP, distal pancreatectomy; IHC, immunohistochemistry; MST, median survival time; N/A, not available; PD, pancreaticoduodenectomy; PR, partial resection; TP, total pancreatectomy; UICC, Union for International Cancer Control 7th edition.
P-values of <0.05 were considered statistically significant; bold type indicates a significant difference.

established by long-term exposure to chemotherapy agents or irradiation. The comparison between each of the resistant cells and the parental cells revealed no significant differences in the mRNA expression of HDAC1, and neither DHOB nor Vorinostat treatments improved the chemosensitivity of chemotherapy-resistant PDAC cells. Furthermore, Vorinostat treatment inhibited the invasiveness of these resistant cells; these results were consistent with the results obtained using clinical samples (Supplementary Figures S3 and S5).

3.3 | The SNAIL expression in clinical samples

To validate the results, we evaluated the SNAIL expression in clinical samples. The expression of SNAIL was detected at the nuclei of all samples; however, the number of SNAIL-positive cells varied widely. Fifty percent of PDAC samples showed >72% SNAIL-positive cells, and the PDAC samples from the HDAC1^{high} group contained numerous SNAIL-positive cells. Moreover, the nuclei of HDAC1-positive cells were also SNAIL-positive (Figure 4B,C). Taken together, these

FIGURE 2 The HDAC1 activity and the epithelial-mesenchymal transition (EMT) in the PDAC cell lines, PSN1, MiaPaCa2, Panc1 and BxPC3. The mean ± standard deviation (SD) is depicted. Significant differences were observed between the values under the horizontal lines (*$P < 0.05$, **$P < 0.01$). HDAC1 activities (A) and the mRNA expression of *HDAC1* (B) in each PDAC cell line. C, The fold change of the number of invasive cells in each PDAC cell line is depicted. The number of BxPC3 cells was set as the control. D, The mRNA expression of the EMT-related genes in each PDAC cell line is depicted; *Cytokeratin 19* (as an epithelial marker), *Vimentin* (as a mesenchymal marker), *SNAIL*, *ZEB1* and *SLUG* (as EMT-related transcriptional factors). Neither *ZEB2* nor *TWIST* were detected in these PDAC cell lines. E, The protein expression in each PDAC cell line was determined by western blotting. The expression level of each protein was normalized by dividing with the corresponding housekeeping protein expression and is depicted below. The expression of HDAC1 in the extracted nuclear proteins is shown in the upper panels. The expression of CK19 in whole cell lysate proteins is shown in the lower panels. In the extracted nuclear proteins, the expression of HDAC1 was highest in PSN1 rather than other PDAC cell lines. In whole cell lysates, the CK19 expression in BxPC3 was highest in 4 PDAC cell lines. F, Immunocytochemistry to detect the expression of HDAC1 and CK 19 in PDAC cells. HDAC1 and CK 19 were stained red or green, respectively. The nuclei stained with Hoechst appear blue. Scale bar: 100 μm. In PSN1 and MiaPaCa2, HDAC1 was highly expressed and CK19 was slightly expressed; these cells had a spindle-like shape. In Panc1 and BxPC3, the HDAC1 expression was low and the CK19 expression was high; these cells had a valvate-like shape


(A)
HDAC1 activity level, fold change
PSN1
MiaPaCa2
Panc1
BxPC3
(B)
HDAC1 mRNA level, fold change
(C)
Number of invasive cells, fold change
(D)
CK19 mRNA level, fold change
Vimentin mRNA level, fold change
SNAIL mRNA level, fold change
ZEB1 mRNA level, fold change
SLUG mRNA level, fold change
(E)
Nuclear proteins
HDAC1
H3
HDAC1 level, fold change
Whole cell lysates
CK19
Actin
CK19 level, fold change
(F)
PSN1
MiaPaCa2
Panc1
BxPC3
Phase contrast
HDAC1
CK19
Hoechst
Merge

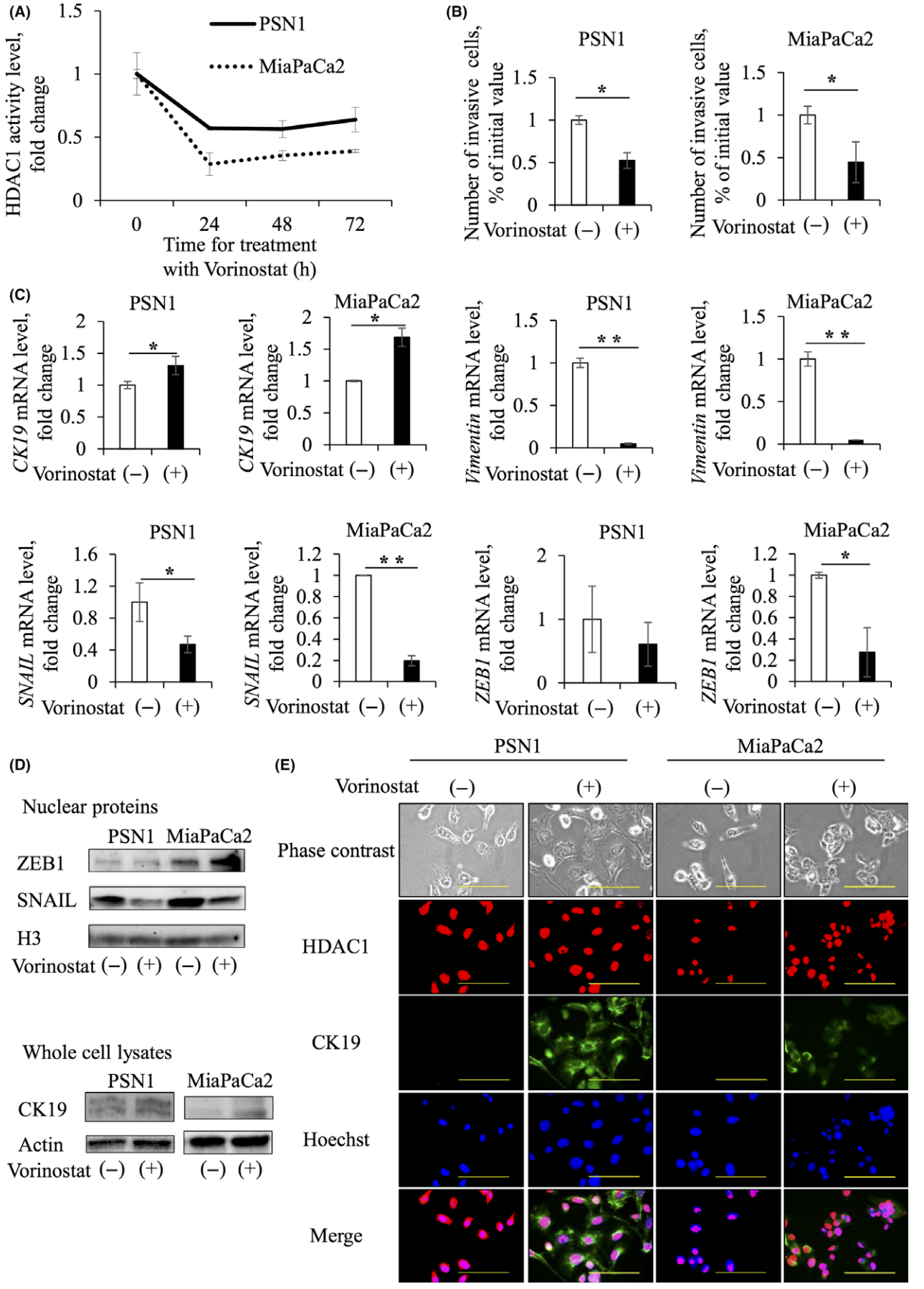

(A)
HDAC1 activity level, fold change
PSN1
MiaPaCa2
Time for treatment with Vorinostat (h)
(B)
Number of invasive cells, % of initial value
PSN1
MiaPaCa2
Vorinostat (–) (+)
(C)
CK19 mRNA level, fold change
Vimentin mRNA level, fold change
SNAIL mRNA level, fold change
ZEB1 mRNA level, fold change
(D)
Nuclear proteins
ZEB1
SNAIL
H3
Whole cell lysates
CK19
Actin
(E)
Vorinostat
Phase contrast
HDAC1
CK19
Hoechst
Merge

FIGURE 3 Treatment of PDAC cells with Vorinostat. The mean ± standard deviation (SD) is shown. Significant differences were observed between the values under the horizontal lines (*$P < 0.05$, **$P < 0.01$). A, The alternation of HDAC1 activity in PSN1 or in MiaPaCa2 with Vorinostat treatment. HDAC1 activity in both PSN1 and MiaPaCa2 was significantly inhibited for 72 h. B, The alternation in the number of invasive cells with Vorinostat treatment. The percentage of the initial change after treatment is shown. Invasiveness in PSN1 and MiaPaCa2 decreased with Vorinostat treatment. C, The alternation of the EMT-related genes with/without Vorinostat treatment in PSN1 and MiaPaCa2. The mRNA expression of *CK19* in both PSN1 and MiaPaCa2 was significantly increased by Vorinostat treatment, and the expression of *Vimentin* in both PSN1 and MiaPaCa2 was significantly decreased. The mRNA expression of *SNAIL* in PSN1 and MiaPaCa2 was significantly decreased by Vorinostat treatment. The mRNA expression of *ZEB1* in MiaPaCa2 was significantly decreased by Vorinostat treatment. D, The protein expression in PSN1 and MiaPaCa2 with/without Vorinostat treatment, as determined by western blotting. The expression of ZEB1 and SNAIL in the extracted nuclear proteins is shown in the upper panels. The expression of CK19 in whole cell lysate proteins was used for the expression levels in the lower panels. In the extracted nuclear proteins, the expression of SNAIL in PSN1 and MiaPaCa2 was decreased by Vorinostat treatment. In whole cell lysates, the CK19 expression in PSN1 and MiaPaCa2 was increased by Vorinostat treatment. E, Immunocytochemistry to detect the expression of HDAC1 and CK 19 in PDAC cells with/without Vorinostat treatment. HDAC1 and CK19 were stained red and green, respectively. The Hoechst-stained nuclei appear blue. Scale bar: 100 μm. The CK19 expression in PSN1 and MiaPaCa2 was increased by Vorinostat treatment

findings suggest that HDAC1 activation induced the migration of PDAC cells to distant organs through the activation of an EMT-related transcriptional factor, SNAIL. Thus, an HDAC1 inhibitor that attenuates SNAIL would be expected to suppress metastasis in PDAC.

4 | DISCUSSION

This study has 3 important findings. First, the expression of HDAC1 was associated with metastasis in PDAC, regardless of whether chemotherapy or radiotherapy was administered. Second, the HDAC1 activity increased the invasion ability, despite the fact that it did not influence resistance to chemotherapy or radiotherapy. Third, an agent targeting HDAC1 suppressed invasiveness through the downregulation of SNAIL. These results indicated that the targeting of HDAC1 has the potential to suppress metastasis in PDAC in the clinical setting.

Previous studies reported that the EMT is pivotal for metastasis in PDAC.[27,28] In the present study, we demonstrated that the expression of HDAC1 was associated with metastasis in PDAC through the EMT. Von Burstin et al[28] also mentioned that HDAC1 plays an essential role during the process of PDAC metastasis; our results were consistent with those of previous reports. However, our study demonstrated that the expression of HDAC1 was not associated with chemosensitivity in PDAC. Although the EMT of the PDAC cells did not show chemoresistance in the present study, several reports have demonstrated that the EMT induced both invasiveness but also chemoresistance in PDAC.[29] This discrepancy may be explained by an epigenetic modulator, HDAC1. HDAC1 could concomitantly target proteins, and certain target proteins may affect chemosensitivity.

Our study demonstrated that HDAC1 inhibitors only target the migration ability; thus, it must be added to the present standard therapies. However, there are no reports on the HDAC activity in PDAC in patients undergoing chemotherapy or radiotherapy. In the present study, we investigated specimens from patients who had been treated with/without preoperative therapy, and found no difference in their expression of HDAC1 (Supplementary Table S3). This led us to hypothesize that HDAC1 inhibitor treatment would work well regardless of the influence of chemotherapy or radiotherapy. Thus, we assumed that unresectable local advanced PDAC could be a suitable target for HDAC inhibitor treatment (as part of a multidisciplinary approach). There are some reports about the evaluation of the HDAC1 expression in clinical samples.[30-32] These reports also suggest that HDAC1 could be a therapeutic target in patients with PDAC.[28,29] However, there is no consensus as to which patients should be treated by HDAC inhibitors in the clinical setting. This is the first study to investigate the possibility of applying HDAC inhibitor treatment for PDAC in the clinical setting.

We reported that the HDAC1 inhibitor suppressed invasiveness, and that SNAIL seemed to play a dominant role in this mechanism. An epigenetic enzyme of HDAC1 could regulate other genes related to invasiveness, and several studies have reported that an epigenetic mechanism governs SNAIL to induce the EMT.[33] SNAIL is one of the SNAG domains, which contain zinc finger proteins, and a number of SNAG-associated histone modification complexes (ie, Sin3A-HDAC1/2, EZH2/-HDAC1/2, LSD1-CoREST and Ajuba-PRMT5) have been identified.[34-38] The manner in which the complexes are orchestrated and assembled at the target chromatin has been reported.[33] Peinado et al[38] state that Snail mediates E-cadherin through the recruitment of the Sin3A/HDAC1/HDAC2 complex in MDCK cells and MCA3D cells. Von Burstin et al[28] report that Snail/HDAC1/HDAC2 repressor complex in PDAC suppresses the EMT regulating metastasis. Although these studies all referred to the regulation of metastasis, our results were not contradictory to them. Moreover, we assumed that a pan-HDAC inhibitor is more effective to this mechanism than an HDAC1-selective inhibitor because it induces downregulations of both HDAC1 and HDAC2.

In our study, we treated cells with Vorinostat, which is a pan-HDAC inhibitor, and DHOB, which is a HDAC1-selective inhibitor. Although the results would be much more convincing with a HDAC1 knockdown experiment, PDAC cells unfortunately lost viability with

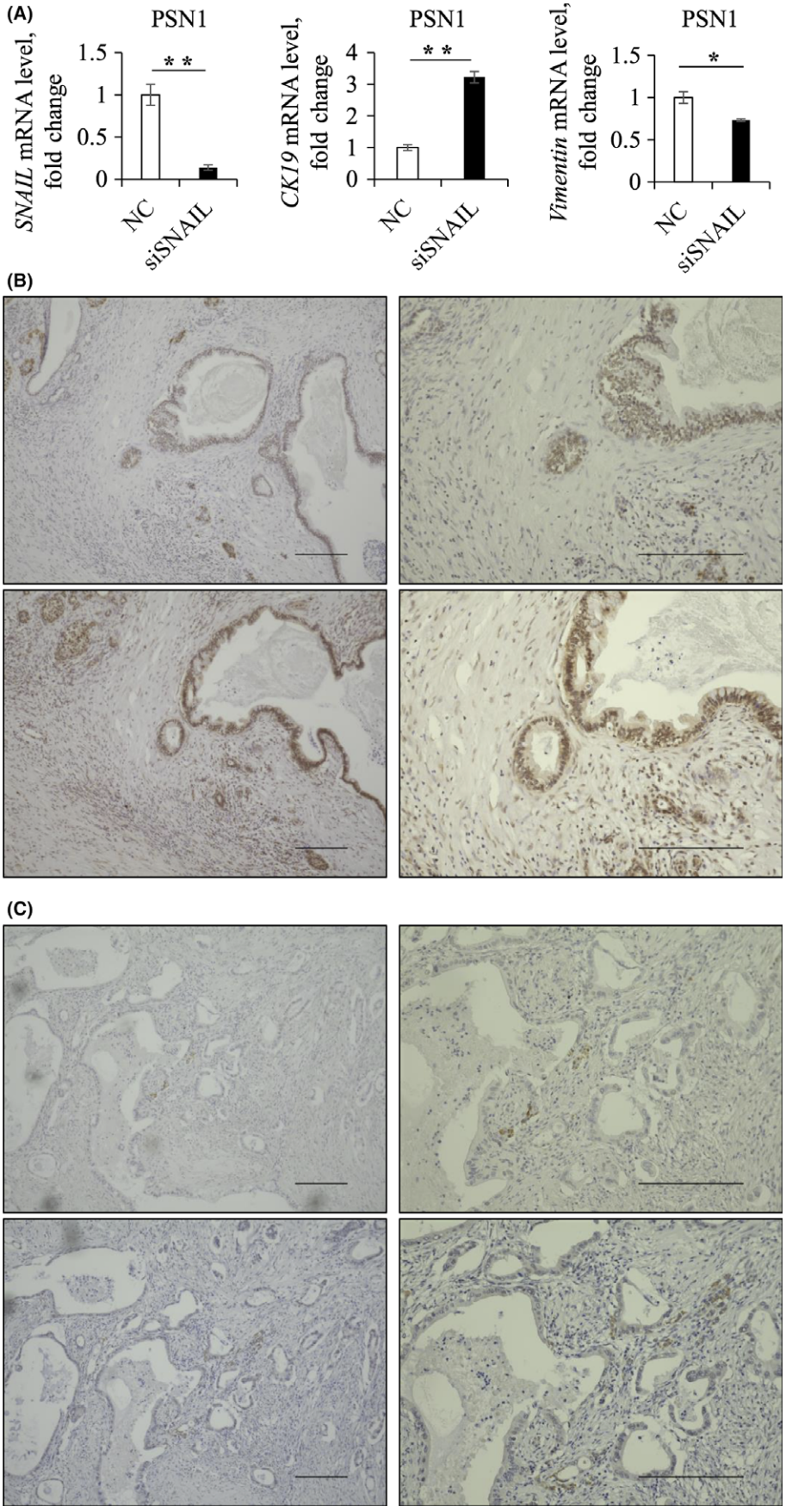

(A)
PSN1
SNAIL mRNA level, fold change
1.5
1
0.5
0
* *
NC
siSNAIL
PSN1
CK19 mRNA level, fold change
4
3
2
1
0
* *
NC
siSNAIL
PSN1
Vimentin mRNA level, fold change
1.5
1
0.5
0
*
NC
siSNAIL
(B)
(C)

FIGURE 4 The Role of *SNAIL* on EMT. The downregulation of *SNAIL* gene in PSN1 and immunohistochemistry to detect HDAC1 and SNAIL in resected PDAC specimens. The mean ± standard deviation (SD) is shown. Significant differences were observed between the values under the horizontal lines (*$P < 0.05$, ** $P < 0.01$). Representative immunohistochemistry for each protein was performed in the same place in serial sections (each column). The right panels show close-up views of the left panels. Scale bar: 200 μm. A, The alternation of the EMT-related mRNA expressions, *SNAIL*, *CK19* and *Vimentin*, in PSN1 with si-*SNAIL* transfection. The expression in PSN with the scramble genes transfection is depicted as negative control (NC). B, The panels show representative examples of specimens with high expression levels of HDAC1 (upper) and SNAIL (lower) (as determined by immunohistochemical staining). C, The panels show representative examples of specimens with low expression levels of HDAC1 (upper) and SNAIL (lower) (as determined by immunohistochemical staining)

HDAC1 knockdown, and, thus, we did not use HDAC1 knockdown cells in our study.

In conclusion, we demonstrated that the HDAC1 expression in PDAC indicated greater migration ability, and that the expression was not influenced by chemotherapy or radiotherapy. The results of the present study suggest that therapy targeting HDAC1 is a promising treatment option for PDAC, and that it would suppress metastasis by inhibiting the EMT.

CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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