




## ORIGINAL ARTICLE

# Aldolase A promotes epithelial-mesenchymal transition to increase malignant potentials of cervical adenocarcinoma

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**Abstract**

Recent studies have revealed that metabolic reprogramming is closely associated with epithelial-mesenchymal transition (EMT) during cancer progression. Aldolase A (ALDOA) is a key glycolytic enzyme that is highly expressed in several types of cancer. In this study, we found that ALDOA is highly expressed in uterine cervical adenocarcinoma and that high ALDOA expression promotes EMT to increase malignant potentials, such as metastasis and invasiveness, in cervical adenocarcinoma cells. In human surgical specimens, ALDOA was highly expressed in cervical adenocarcinoma and high ALDOA expression was correlated with lymph node metastasis, lymphovascular infiltration, and short overall survival. Suppression of ALDOA expression significantly reduced cell growth, migration, and invasiveness of cervical cancer cells. Aldolase A expression was partially regulated by hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ). Shotgun proteome analysis revealed that cell-cell adhesion-related proteins were significantly increased in ALDOA-overexpressing cells. Interestingly, overexpression of ALDOA caused severe morphological changes, including a cuboidal-to-spindle shape shift and reduced microvilli formation, coincident with modulation of the expression of typical EMT-related proteins. Overexpression of ALDOA increased migration and invasion in vitro. Furthermore, overexpression of ALDOA induced HIF-1 $\alpha$ , suggesting a positive feedback loop between ALDOA and HIF-1 $\alpha$ . In conclusion, ALDOA is overexpressed in cervical adenocarcinoma and contributes to malignant potentials of tumor cells through modulation of HIF-1 $\alpha$  signaling. The feedback loop between ALDOA and HIF-1 $\alpha$  could become a therapeutic target to improve the prognosis of this malignancy.

**KEYWORDS**

aldolase A, cervical adenocarcinoma, epithelial-mesenchymal transition, hypoxia-inducible factor-1 $\alpha$ , metabolic reprogramming

Yuki Saito and Akira Takasawa are contributed equally to this work.

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## 1 | INTRODUCTION

The incidence of uterine cervical adenocarcinoma has been dramatically increasing worldwide, predominantly in young women, despite the fact that the incidence of squamous cell carcinoma (SCC) has been decreasing.<sup>1-7</sup> It has been shown that adenocarcinoma has a worse prognosis than that of SCC at the same stage and with the same tumor size.<sup>8-12</sup> The main reasons for the worse prognosis are a higher rate of metastases and resistance to chemoradiotherapy.<sup>3</sup> Consequently, a novel therapeutic strategy is needed to improve the outcome of cervical adenocarcinoma.<sup>13-16</sup>

Fructose-bisphosphate aldolase is one of the key glycolytic enzymes that catalyzes the reversible reaction of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Aldolase is ubiquitously distributed in all organs and/or cells and it plays an essential role in ATP biosynthesis, especially in a glycolytic pathway under anaerobic conditions.<sup>17</sup>

Aldolase A (ALDOA) is overexpressed in various cancers<sup>18</sup> including squamous cell lung cancer,<sup>19,20</sup> hepatocellular carcinoma,<sup>21</sup> colonic cancer,<sup>22</sup> osteosarcoma,<sup>23,24</sup> and pancreatic cancer.<sup>25</sup> In recent years, some researchers have reported that overexpression of ALDOA is involved in malignant behaviors of various cancer cells.<sup>19,23,26-28</sup> However, there is no information about the relationship between expression of ALDOA and malignant behavior of cervical adenocarcinoma cells.

In this study, we examined the expression of ALDOA in cervical adenocarcinoma tissues by immunohistochemistry and the relationships between ALDOA expression and clinicopathologic features. We also investigated the importance of ALDOA expression in the malignant behavior of uterine cervical cancer cells. This is the first report showing that ALDOA is overexpressed in cervical adenocarcinoma specimens and that overexpression of ALDOA enhances malignant behavior through induction of epithelial-mesenchymal transition (EMT).

## 2 | MATERIALS AND METHODS

### 2.1 | Immunohistochemistry and immunohistochemical analysis of surgical specimens

Specimens of 53 cases of cervical adenocarcinoma including adenocarcinoma in situ (AIS) obtained by surgical resection during the period from 2004 to 2012 were retrieved from the pathology file of Sapporo Medical University Hospital (Sapporo, Japan). This study was approved by the Institutional Review Board of Sapporo Medical University (IRB study number 302-197). Written informed consent was obtained from each patient who participated in the investigation. As controls, adjacent nonneoplastic regions were examined as normal tissues (n = 40). Clinicopathologic features of the patients were described previously.<sup>13</sup> Immunohistochemistry was carried out with anti-ALDOA (1:500; Sigma-Aldrich) as described previously.<sup>29</sup> Surgical specimen staining patterns were scored as follows: score

0, no reactivity or cytosolic reactivity in less than 10% of tumor cells; score 1+, faint/almost no cytosolic reactivity in 10% or more of tumor cells; score 2+, weak to moderate cytosolic reactivity in 10% or more tumor cells; and score 3+, moderate to strong cytosolic reactivity in 10% or more of tumor cells. For statistical purposes, samples with scores 0 and 1+ were considered negative, and those with scores 2+ and 3+ were considered positive. When evaluating the slides, the observers (YS, MM, and AT) were blinded to the clinical data. Discordant cases were discussed, and a consensus was reached.

### 2.2 | Cell culture and treatment

The human cervical adenocarcinoma cell lines Hela229 and OMC4 were purchased from RIKEN Bio-Resource Center (Tsukuba, Japan) and HCA1 was from JCRB Cell Bank (Osaka, Japan). The human cervical adenocarcinoma cell line CAC-1 and TMCC-1 were provided by our colleague Dr Hayakawa.<sup>30,31</sup> Cells were maintained as described previously.<sup>14</sup> Human ALDOA-specific siRNAs (5'-GUGUCAUCCUCUUCCAUGA-3' and 5'-GUCAUCCUCUUCCAUGAGA-3'), human hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ )-specific siRNAs (5'-GAAUUACGUUGUGAGUGGU-3' and 5'-GAUUAACUCAGUUUGAACU-3') and siRNA universal negative control were purchased from Sigma-Aldrich. Transfection of siRNA was carried out by using RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The coding sequence of human ALDOA gene was obtained by RT-PCR by using total RNA of HCA1 cells as a template and using primers 5'-CATAAGCTTCCTACCAATATCCAGCACTG-3' and 5'-CGGAATCTTAATAGGCGTGGTTAGAGA-3'. The amplified fragment was cloned between the *Hind*III site and *Eco*RI site of pCMV10-3xFLAG vector (Thermo Fisher Scientific). To obtain stable clones overexpressing ALDOA, the 3xFLAG-ALDOA expressing vector was transfected in HCA-1 cells by using Lipofectamine 3000 (Thermo Fisher Scientific) followed by selection with 800  $\mu$ g/mL neomycin for 14 days. The neomycin-resistant cells were then seeded in 96-well plates at a density of 1 cell/well to obtain single clones. The ALDOA-overexpressing cells (FLAG-ALDOA cells) were screened by western blot by using anti-FLAG (M2) Ab. For hypoxia incubation, cells were maintained at 2% O<sub>2</sub>, 5% CO<sub>2</sub>, and 93% N<sub>2</sub> at 37°C, using an automatic multigas incubator (IncuSafe, MCO-5AC-PE; PHC). Primary Ab information is listed in Table S1.

### 2.3 | Cellular ATP detection assay

A luminescence ATP detection assay system, ATPlite 1step (Perkin Elmer, Boston, MA), was used to measure the level of cellular ATP according to the manufacturer's instructions. Briefly, subconfluent cells (6-cm dish) were trypsinized and seeded in a white-wall, 96-well plate (Corning) at a concentration of 10<sup>3</sup> cells/100  $\mu$ L medium per well. ATPlite 1step reagent (100  $\mu$ L) was added and then the luminescence was measured on

a Varioskan LUX multimode microplate reader (Thermo Fischer Scientific).

## 2.4 | Cell proliferation assay

In the WST-8 assay, cells were seeded in 96-well plates and their viability was assessed at 24 hours after incubation by using a CCK-8 (Dojindo Laboratories) according to the manufacturer's instructions. Absorbance at a wavelength of 450 nm was measured by using the Varioskan LUX multimode microplate reader (Thermo Fischer Scientific). Colony forming assay was carried out as described previously.<sup>32</sup> Each experiment was independently repeated 3 times.

## 2.5 | Immunocytochemistry of cell blocks and immunofluorescence microscopy

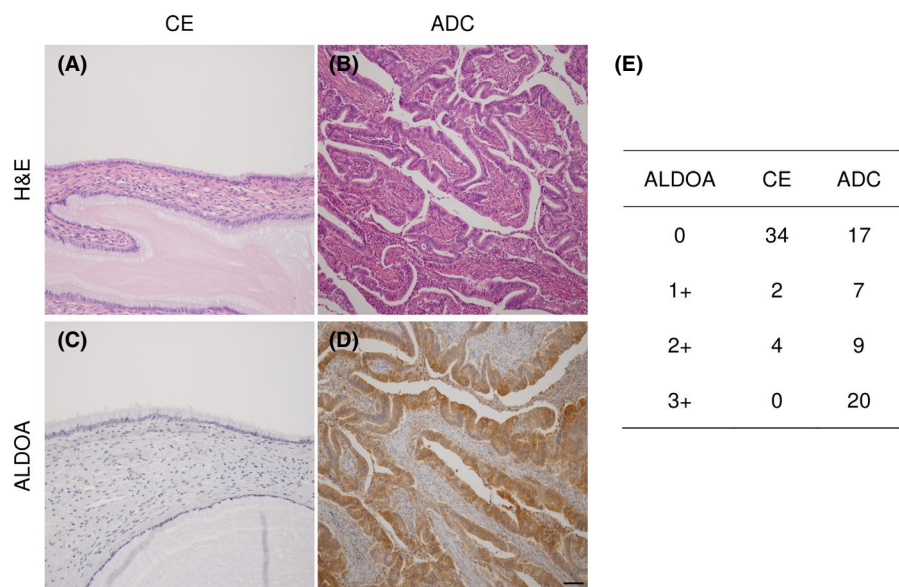
Cell blocks were prepared by using the sodium alginate method as described previously.<sup>33</sup>

## 2.6 | Cell migration and invasion assays

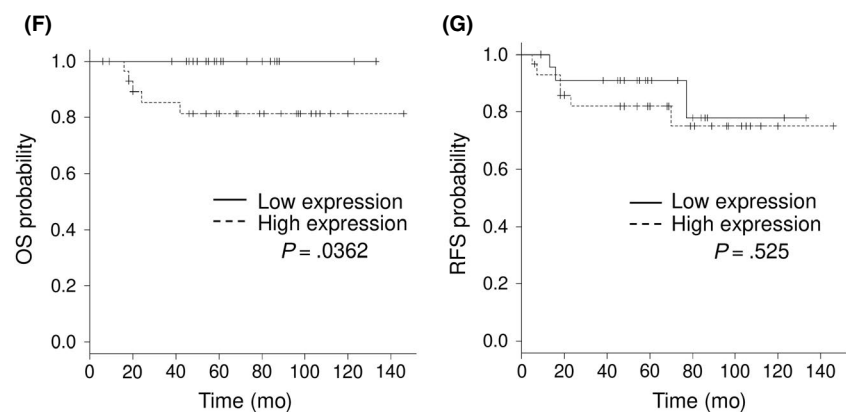
The migration assay was carried out using Transwell (Corning; 8- $\mu$ m pore polycarbonate membrane insert) in 24-well dishes and the invasion assay was performed using Biocoat Matrigel (Corning; pore size, 8- $\mu$ m) as described previously.<sup>34</sup> Each experiment was independently repeated 3 times.

## 2.7 | Peptide preparation

Protein was extracted using a phase transfer surfactant method as described previously.<sup>35</sup> Briefly, cells were lysed with 50 mmol/L ammonium bicarbonate containing 12 mmol/L sodium deoxycholate, 12 mmol/L sodium N-lauroylsarcosinate, and EDTA-free protease inhibitor cocktail (Roche Diagnostics). Lysates were heated at 95°C for 5 minutes, sonicated, and then centrifuged at 19 000 g for 15 minutes at room temperature. Supernatants were collected and protein concentration was measured by a reducing agent compatible version of the Pierce microplate BCA protein assay kit (Thermo Fischer Scientific). Fifty micrograms of protein lysate was reduced with 10 mmol/L DTT, alkylated with 20 mmol/L



**FIGURE 1** Aldolase A (ALDOA) is highly expressed in surgical specimens of cervical neoplasms. A-D, Immunohistochemistry of ALDOA in surgical specimens of human cervical adenocarcinoma. ALDOA was strongly expressed in adenocarcinoma (ADC), whereas it was undetectable in the nonneoplastic cervical epithelium (CE). Scale bar = 50  $\mu$ m. E, Immunoreactive intensities of ALDOA in CE and ADC. F, G, Kaplan-Meier estimates of overall survival (OS) and relapse-free survival (RFS) of patients with cervical adenocarcinoma. High expression level of ALDOA was significantly correlated with worse OS ( $P = .0362$ )



iodoacetamide, and then diluted with 50 mmol/L ammonium bicarbonate, followed by digestion with 1:50 (w/w) trypsin for 18 hours at 37°C. After digestion, an equal volume of ethyl acetate containing 1% TFA was added. The mixture was vortexed for 1 minute and centrifuged at 15 600 g for 3 minutes. The upper phase was discarded. The lower aqueous phase was concentrated under vacuum and then desalted by GL-Tip SDB according to the instructions of the manufacturer (GL Science).

## 2.8 | Proteome analysis

Samples were dissolved in 0.1% formic acid and loaded into a nano-flow UHPLC (Easy-nLC 1000 system; Thermo Fisher Scientific) online-coupled to an Orbitrap mass spectrometer equipped with a nanospray ion source (Q-Exactive Plus; Thermo Fisher Scientific). Samples were separated by using a 75  $\mu\text{m} \times 20$  cm capillary column with a particle size of 3  $\mu\text{m}$  (NTCC-360; Nikkyo Technos) by applying a linear gradient ranging from 5% to 35% buffer B (100% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min for 120 minutes. In mass spectrometry analysis, survey scan spectra were acquired at a resolution of 70 000 at 200 m/z with a target value of 3e6 ions, ranging from 350 to 2000 m/z with charge states between 1+ and 4+. We applied a data-dependent top 10 method that generates high-energy collision dissociation fragments for the 10 most intense precursor ions per survey scan. The tandem mass spectrometry (MS/MS) resolution was 17 500 at 200 m/z with a target value of 1e5 ions.

For MS/MS data analysis, we used the Sequest HT (Thermo Fisher Scientific) and Mascot version 2.5 (Matrix Science) algorithms embedded in the Proteome Discoverer 2.2 platform (Thermo Fisher Scientific), and the peak lists were searched against the UniProt human databases. The tolerance of precursor ions and fragment ions were set to 10 ppm and 0.02 Da, respectively.

## 2.9 | Scanning electron microscopy

For scanning electron microscopy, cells grown on coverslips were fixed with 2.5% glutaraldehyde in PBS overnight at 4°C. After several rinses with PBS, the cells were postfixed in 1% OsO<sub>4</sub> in PBS at 4°C for 3 hours and washed with distilled water, followed by dehydration through a graded series of ethanol and freeze drying. Samples were sputter-coated with platinum and examined under a scanning electron microscope (S4300; HITACHI) operating at 10 kV.

## 2.10 | Statistical analysis

The measured values are presented as mean  $\pm$  SD. Data were analyzed and compared using the unpaired 2-tailed Student's *t* test, Fisher's exact test, and Kruskal-Wallis test. Survival rates were

calculated by the Kaplan-Meier method and compared by the log-rank test. Statistical significance was accepted when  $P < .05$  (\* $P < .05$  and \*\* $P < .01$ ). All statistical analyses were undertaken with EZR software.<sup>36</sup>

## 3 | RESULTS

### 3.1 | Expression profiles of ALDOA in surgical specimens of cervical adenocarcinoma

First, we undertook the immunohistochemistry of surgical specimens for ALDOA in uterine cervical adenocarcinomas. In nonneoplastic cervical gland tissues, immunostaining of ALDOA was faint or absent (Figure 1A,C). In contrast, strong and diffuse immunostaining of ALDOA was observed in the cytoplasm in adenocarcinoma (ADC) (Figure 1B,D). On the basis of the immunoreactive intensity, the patients were classified into 2 groups: a high ALDOA expression group (intensity of 2+ or 3+) and a low ALDOA expression group (intensity of 1+ or 0). Twenty-four (55.9%) of 43 ADC cases had high ALDOA expression. Those cases included 18 cases (41.9%) and 6 cases (14.0%) with intensities of 3+ and 2+, respectively. Five (50.0%) of 10 AIS cases had high ALDOA expression. They included 2 cases (20.0%) and 3 cases (30.0%) with intensities of 3+ and 2+,

**TABLE 1** Clinicopathologic parameters of cervical adenocarcinoma

	N	ALDOA		P value
		0, 1+	2+, 3+	
Diameter (mm)				
<=40	41	21	20	.3460
>40	12	3	9	
UICC				
0	10	5	5	.1050
I	32	17	15	
II	5	2	3	
III	6	0	6	
Tumor factor				
pT0	10	5	5	.3710
pT1	33	17	16	
pT2	9	2	7	
pT3	1	0	1	
N factor				
N0	47	24	23	.0266
N1	6	0	6	
Lymphovascular infiltration				
Negative	38	22	16	.0051
Positive	15	2	13	

ALDOA, aldolase A.

respectively. Thus, ALDOA expression was high in 29 (54.7%) of the 53 AIS/ADC cases, including 20 cases (37.7%) with an intensity of 3+ and 9 cases (17.0%) with an intensity of 2+ (Figure 1E).

### 3.2 | Correlations between expression of ALDOA and clinicopathologic features

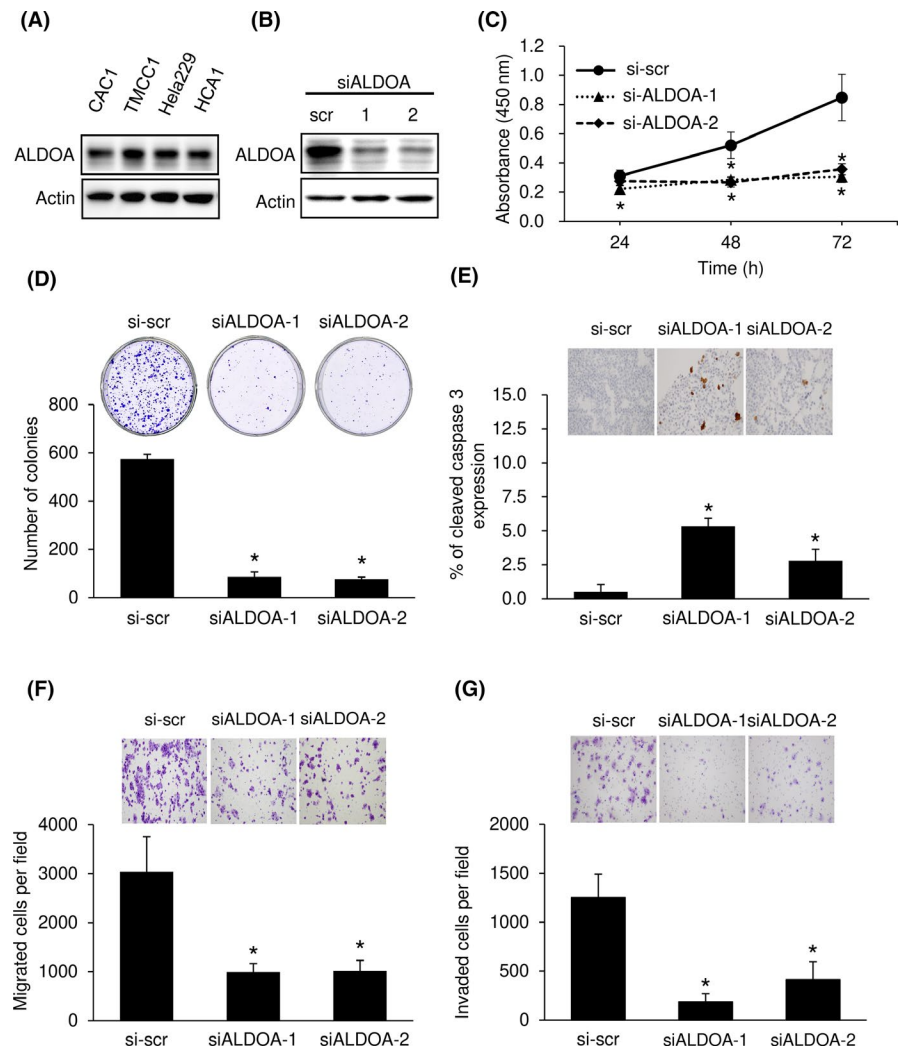
We next evaluated the relationships of ALDOA expression intensity with clinicopathologic features and outcome of cervical adenocarcinoma. As shown in Table 1, high ALDOA expression was significantly correlated with lymph node metastasis ( $P = .0266$ ) and lymphovascular infiltration ( $P = .0051$ ). There was no significant correlation between ALDOA expression and histological type, diameter, UICC stage, or tumor factor. The relationships between ALDOA expression and relapse-free survival and overall survival of patients with cervical adenocarcinoma were assessed by using the Kaplan-Meier method. Overall survival was significantly shorter in patients with high ALDOA expression than in patients with low ALDOA expression (Figure 1F), but there was no correlation between ALDOA expression and relapse-free survival (Figure 1G). These results

suggest that ALDOA is not only highly expressed in cervical adenocarcinoma but also might play a role in the development of cervical adenocarcinoma.

### 3.3 | Knockdown of ALDOA attenuates the malignant potential of cervical adenocarcinoma cells

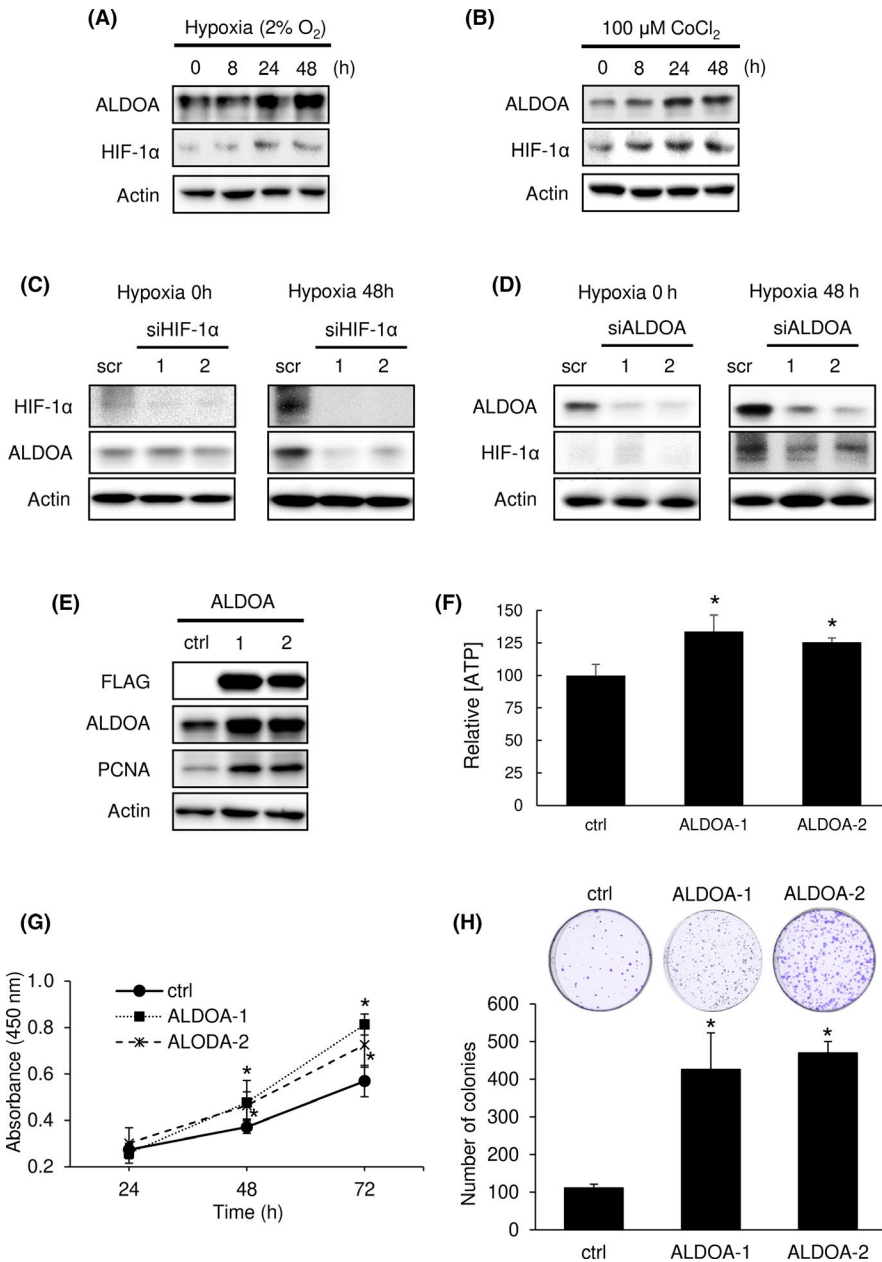
To examine the effects of ALDOA expression on tumor cells, we investigated the expression patterns of ALDOA in human uterine cervical cancer cell lines HCA1, HeLa229, TMCC1, and CAC1. In all of the cell lines examined, ALDOA was constitutively expressed (Figure 2A). Among the cell lines, the well-differentiated type of cervical adenocarcinoma cell line HCA1 was mainly used for subsequent experiments because the cell line retains some epithelial properties, including morphologically intact adherence junctions and tight junctions, and does not carry human herpes viruses.<sup>14</sup>

First, we suppressed the expression of ALDOA by ALDOA-specific siRNAs (Figures 2B, S1A and S2). In a WST-8 cell proliferation assay, the percentage of proliferative cells in ALDOA knockdown (KD) cells was significantly lower than that in control cells treated with scrambled siRNA (Figures 2C and S1B). In a



**FIGURE 2** Knockdown (KD) of aldolase A (ALDOA) inhibits proliferation, migration, and invasion of cervical adenocarcinoma cells. A, ALDOA was expressed in all of the tested cervical cancer cell lines. B, Western blot analysis. Knockdown of ALDOA expression was achieved by ALDOA-specific siRNAs compared to scrambled siRNA (scr) in HCA1 cells. C, D, ALDOA siRNAs significantly reduced the proliferation ability of HCA1 cells in WST-8 (C) and colony formation (D) assays. E, Immunohistochemistry of anti-cleaved caspase-3 (apoptosis marker) Ab in cell block samples. F, Transwell migration assay. ALDOA-KD significantly inhibited migration of HCA1 cells. G, Matrigel invasion assay. ALDOA-KD significantly inhibited invasion of HCA1 cells





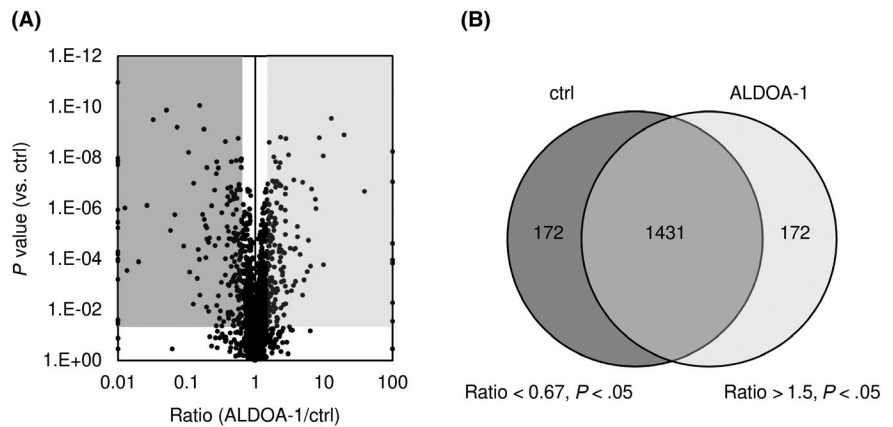
**FIGURE 3** Aldolase A (ALDOA) is induced by hypoxic conditions and overexpression of ALDOA enhances proliferation of cervical adenocarcinoma cells. A, B, ALDOA expression was induced by hypoxic condition (2% O<sub>2</sub>) and by treatment with 100 μmol/L CoCl<sub>2</sub>, a chemical inducer of hypoxia-inducible factor-1α (HIF-1α), in a time-dependent manner in HCA1 cells. C, D, HIF-1α siRNAs (siHIF-1α) significantly reduced ALDOA induction (C), whereas ALDOA-siRNAs did not affect HIF-1α induction (D) under hypoxic conditions. E, 3xFLAG-tagged ALDOA-overexpressing stable clones (ALDOA-1 and -2 cells) were established by using HCA1 cells. Expression of proliferating cell nuclear antigen (PCNA) was induced by ALDOA overexpression. F, The amount of intracellular ATP was significantly larger in ALDOA-overexpressing HCA1 cells than in control cells. G, H, ALDOA overexpression significantly promoted the proliferation ability of HCA1 cells in WST-8 (G) and a colony formation (H) assays. A-E, Western blot analysis

colony formation assay, the number of colonies was significantly smaller for ALDOA-KD cells than for control cells (Figures 2D and S1C). In addition, a large number of cleaved caspase-3-positive cells, an indicator of apoptosis, was observed for ALDOA-KD cells compared with control cells (Figure 2E). These results indicated that ALDOA-KD inhibits cell proliferation, partially due to increased apoptosis, in cervical adenocarcinoma cells. In a migration assay using a Transwell membrane, the number of ALDOA-KD cells migrating through the membrane was significantly smaller than that of control cells (Figures 2F and S1D). Also, in an invasion assay using a Matrigel matrix-coated chamber, the number of ALDOA-KD cells penetrating through the matrix was significantly smaller than that of control cells (Figure 2G). These results indicate that ALDOA expression contributes to the malignant potential of cervical adenocarcinoma cells.

### 3.4 | Aldolase A is induced by hypoxic conditions in cervical adenocarcinoma cells

Next, we examined the factor responsible for increased expression of ALDOA. We hypothesized that ALDOA expression might be induced by hypoxia, as aldolase is well known to be involved in the anaerobic glycolytic pathway.<sup>37</sup> As expected, ALDOA expression was induced together with expression of HIF-1α at 24 hours after culturing cells under hypoxic conditions (2% O<sub>2</sub>) (Figure 3A). Under hypoxic conditions, KD of HIF-1α inhibited induction of both HIF-1α and ALDOA (Figure 3C), whereas knockdown of ALDOA inhibited ALDOA expression but did not affect HIF-1α (Figure 3D), indicating that ALDOA induction is mediated by HIF-1α under hypoxic conditions. Aldolase A expression was also induced by treatment with 100 μmol/L CoCl<sub>2</sub>, a chemical inducer of HIF-1α, in a time-dependent

**FIGURE 4** Comparable proteome analysis of aldolase A (ALDOA)-overexpressing cells and control cells. A, B, Volcano plot (A) and Venn map (B) of proteins that were identified by mass spectrometry-based proteomic analysis from control HCA1 cells (ctrl) and FLAG-tagged ALDOA-overexpressing HCA1 cells (ALDOA-1). A total of 1775 proteins were identified. C, Top 5 enriched gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genome (KEGG) pathways assigned for downregulated proteins in ALDOA-1 cells. False discovery rate (FDR) value was considered as statistically significant when it was less than .05



**(C)** GO terms and KEGG pathways for downregulated proteins in ALDOA-1 cells

Category	GO.ID/BP	GO term	Count	Fold enrichment	FDR
Biological process	GO:0098609	Cell-cell adhesion	18	6.80	2.0E-08
	GO:0006564	L-serine biosynthetic process	3	76.79	9.1E-03
	GO:0016032	Viral process	11	3.77	1.1E-02
	GO:0071681	Cellular response to indole-3-methanol	3	61.43	1.5E-02
	GO:0031532	Actin cytoskeleton reorganization	5	10.89	1.8E-02
Cellular component	GO:0070062	Extracellular exosome	81	3.07	1.6E-21
	GO:0005829	Cytosol	78	2.50	4.3E-15
	GO:0005913	Cell-cell adherens junction	23	7.59	4.5E-12
	GO:0005737	Cytoplasm	90	1.84	8.1E-10
	GO:0005925	Focal adhesion	21	5.72	9.6E-09
Molecular function	GO:0098641	Cadherin binding involved in cell-cell adhesion	24	8.27	1.9E-13
	GO:0019901	Protein kinase binding	16	4.25	7.6E-05
	GO:0000287	Magnesium ion binding	11	5.39	5.3E-04
	GO:0005515	Protein binding	113	1.28	1.1E-03
	GO:0005198	Structural molecule activity	11	4.45	2.6E-03
KEGG pathways	hsa01130	Biosynthesis of antibiotics	12	4.53	1.5E-03
	hsa01100	Metabolic pathways	31	2.03	2.3E-03
	hsa05100	Bacterial invasion of epithelial cells	7	6.57	7.4E-03
	hsa00260	Glycine, serine and threonine metabolism	5	9.38	2.2E-02
	hsa05412	Arrhythmogenic right ventricular cardiomyopathy	6	6.56	2.4E-02

manner (Figure 3B). These results suggest that hypoxic conditions might be associated with increased ALDOA expression through HIF-1 $\alpha$  in cervical adenocarcinoma cells.

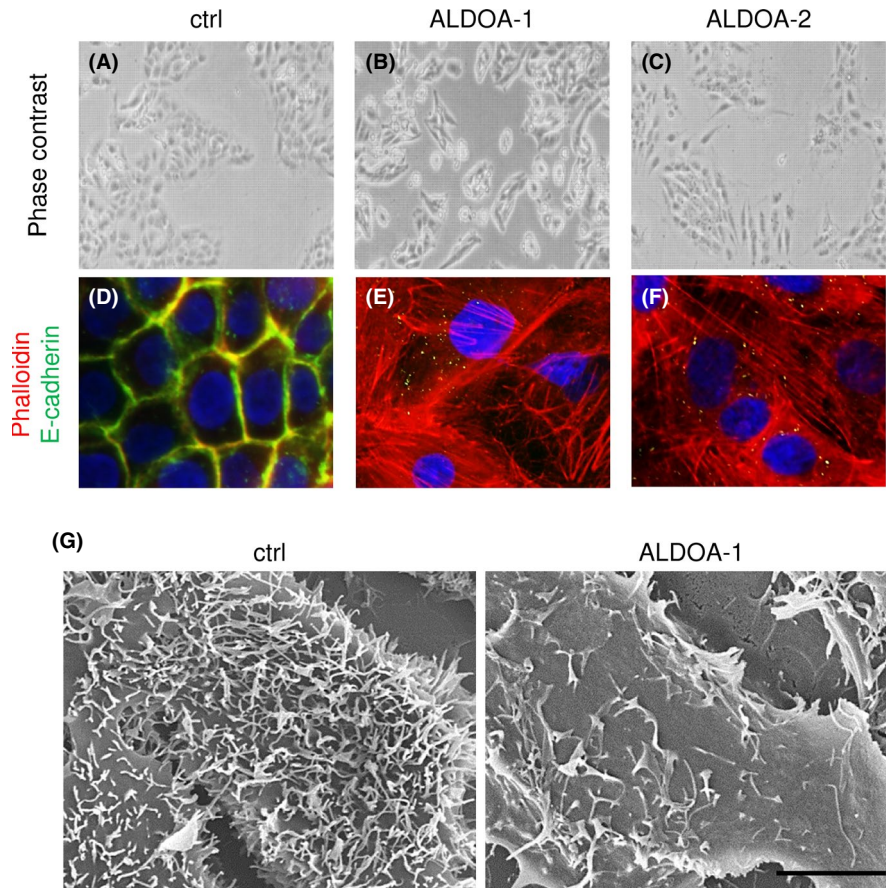
### 3.5 | Overexpression of ALDOA enhances the proliferation of cervical adenocarcinoma cells

To further analyze the effect of ALDOA overexpression, we established stable clones expressing 3xFLAG-tagged ALDOA, hereinafter referred to as FLAG-ALDOA cells (Figure 3E). As expected from the role of ALDOA, the amount of cellular ATP was significantly larger in FLAG-ALDOA cells than in control cells (Figure 3F), indicating that ATP synthesis was increased by ALDOA overexpression. In western blot analysis, these clones showed enhancement of the expression of proliferating cell nuclear antigen, an indicator of cell proliferation (Figure 3E). In a WST-8 cell proliferation assay, overexpression of ALDOA significantly enhanced the proliferation of HCA1 cells

(Figure 3G). In a colony formation assay, the number of colonies was significantly larger in FLAG-ALDOA cells than in control cells (Figure 3H). These results indicate that ALDOA overexpression enhances the proliferation of cervical adenocarcinoma cells.

### 3.6 | Association between ALDOA overexpression and downregulation of cell-cell adhesion molecules was revealed by proteomic analysis

To clarify the role of ALDOA overexpression, we undertook comparative shotgun proteomic analyses. Protein samples from FLAG-ALDOA cells and control cells were digested by trypsin, and the digested peptides were subjected to MS. A label-free quantitation method was used to compare protein expression patterns between FLAG-ALDOA cells and control cells. To ensure the quality and precision of data, each sample measurement was independently repeated



**FIGURE 5** Aldolase A (ALDOA) overexpression leads to epithelial-mesenchymal transition-like morphological alterations in cervical adenocarcinoma cells. A-C, Phase-contrast microscopic images of cell cultures. Overexpression of ALDOA caused morphological changes of cells from an oval shape to a spindle shape. D-F, Immunofluorescence staining of E-cadherin (green) and phalloidin (red). Expression of E-cadherin (green) was significantly suppressed by ALDOA overexpression. Phalloidin staining (red) shows that stress fiber formation was induced by ALDOA overexpression. G, Scanning electron microscopy. The formation of microvilli was suppressed by ALDOA overexpression. Scale bar = 10  $\mu\text{m}$  (C, F), 5  $\mu\text{m}$  (G). ctrl, control

4 times. A total of 1775 unique proteins were identified (Data S1); of these, 172 proteins were upregulated (ratio  $> 1.5$ ,  $P < .05$ ; Table S2) and 172 proteins were downregulated in FLAG-ALDOA cells (ratio  $< 0.67$ ,  $P < .05$ ; Figure 4A,B and Data S1). Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway analyses revealed that enriched GO terms for downregulated proteins were predominantly associated with cell-cell adhesion terms including cell-cell adhesion (GO:0098609), actin cytoskeleton reorganization (GO:0031532), cell-cell adherens junction (GO:0005913), focal adhesion (GO:0005925), and cadherin binding involved in cell-cell adhesion (GO:0098641) (Figure 4C and Data S2). As shown in Data S2, adherens junction molecules ( $\alpha$ -catenin and  $\beta$ -catenin) and a tight junction molecule (junctional adhesion molecule-A [JAM-A]) were included in the list of downregulated proteins in FLAG-ALDOA cells. Listed proteins in each GO term are shown in Data S2. Enriched GO terms for upregulated proteins are shown in Table S2. The results suggest that ALDOA overexpression could influence cell-cell adhesion of cervical adenocarcinoma cells.

### 3.7 | Aldolase A overexpression leads to EMT-like morphological alteration in cervical adenocarcinoma cells

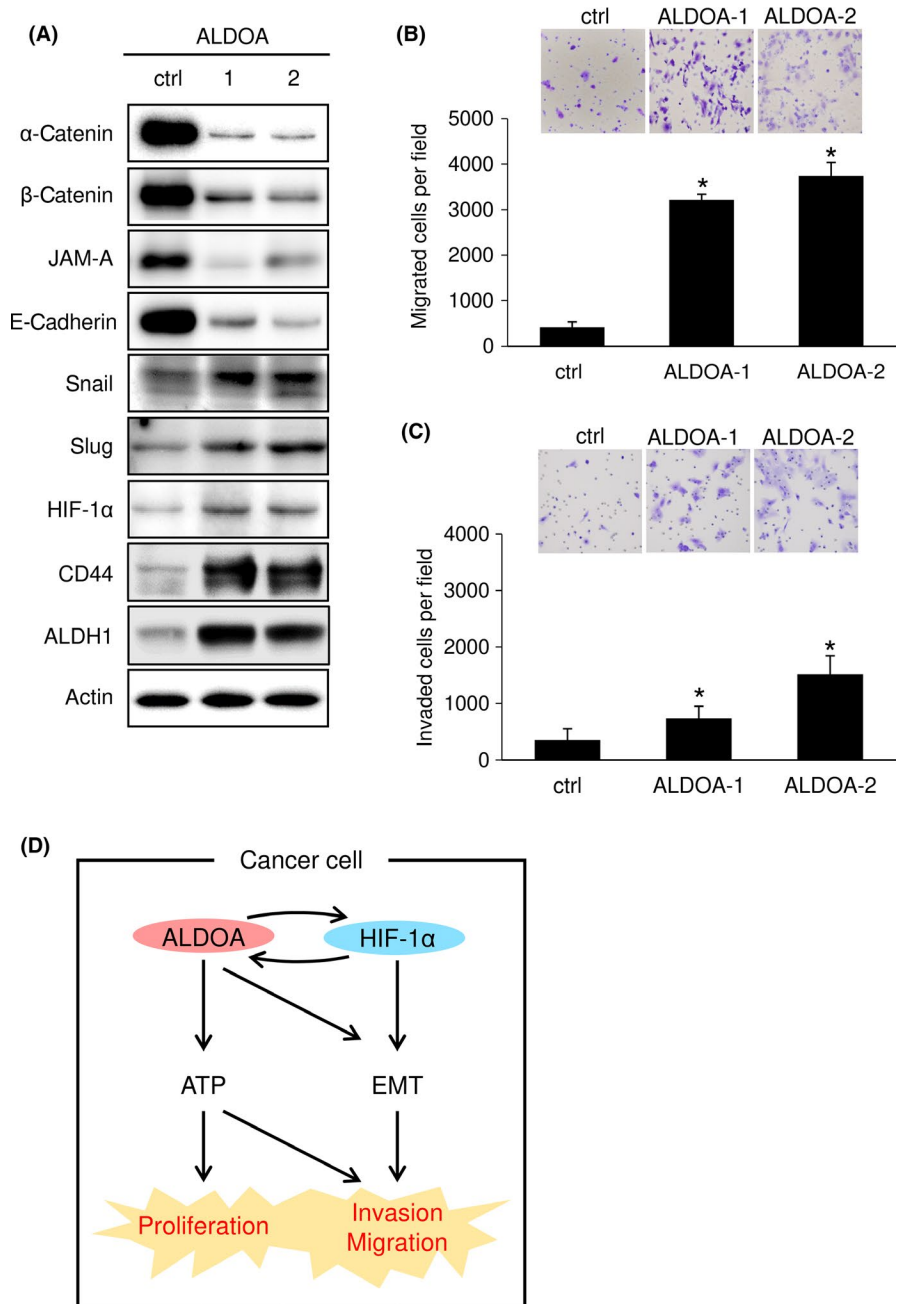
The results of proteomic analysis indicated that ALDOA overexpression causes a comprehensive change in cell-cell adhesion-associated proteins,

evoking EMT. To determine whether ALDOA overexpression is associated with such a phenomenon, we undertook cell-based assays to assess the characteristics of EMT. Phase-contrast microscopy revealed that overexpression of ALDOA (ALDOA-1 and ALDOA-2) caused morphological changes from an oval-cuboidal shape into a spindle shape, resembling a mesenchymal cell-like shape (Figure 5A-C). In immunofluorescence, immunoreactivity of E-cadherin, one of the most important molecules of cell-cell adhesion, was significantly suppressed in FLAG-ALDOA cells (Figure 5D-F). Fluorescein-labeled phalloidin staining revealed that overexpression of ALDOA induced actin stress fiber formation, which is generally observed during the EMT process (Figure 5D-F). Ultrastructural analysis showed that the formation of microvilli was reduced in FLAG-ALDOA cells compared to that in control cells (Figure 5G). These results indicate that ALDOA overexpression caused EMT-like perturbation of cell-cell adhesion and reduction of microvilli formation.

In addition to morphological changes, we examined alteration of molecular expression and phenotypic changes, including migration and invasiveness. Western blot analysis confirmed decreased levels of cell-cell adhesion-associated proteins, including E-cadherin,  $\alpha$ -catenin,  $\beta$ -catenin, and JAM-A, and increased levels of the EMT regulatory proteins Snail, Slug, and HIF-1 $\alpha$  in FLAG-ALDOA cells, which are hallmarks of EMT (Figure 6A). Migration and invasiveness, well-known phenotypes associated with EMT, were also affected by ALDOA overexpression. In a migration assay using a Transwell membrane, the number of cells penetrating through the membrane was significantly large in FLAG-ALDOA cells than



**FIGURE 6** Aldolase A (ALDOA) overexpression leads to epithelial-mesenchymal transition (EMT)-like alterations in cervical adenocarcinoma cells. A, ALDOA overexpression suppressed the expression of cell-cell adhesion-associated proteins ( $\alpha$ -catenin,  $\beta$ -catenin, junctional adhesion molecule-A [JAM-A], and E-cadherin) and increased the expression of EMT regulatory proteins (Snail, Slug, and hypoxia-inducible factor-1 $\alpha$  [HIF-1 $\alpha$ ]) in HCA1 cells. Western blot analysis. B, C, Transwell migration assay and Matrigel invasion assay. ALDOA overexpression significantly promoted migration (B) and invasion (C) of HCA1 cells. D, Illustration showing that ALDOA and HIF-1 $\alpha$  form a positive feedback loop to promote malignant potentials of cervical adenocarcinoma cells. ctrl, control



in control cells (Figure 6B). In an invasion assay using a Matrigel matrix-coated chamber, the number of cells invading through the matrix was significantly larger in FLAG-ALDOA cells than in control cells (Figure 6C). Of note, levels of CD44 and aldehyde dehydrogenase 1 (ALDH1) were increased in FLAG-ALDOA cells (Figure 6A).

#### 4 | DISCUSSION

This is the first study showing that a high level of ALDOA expression plays a significant role in cervical adenocarcinoma. An immunohistochemical study clearly showed that high ALDOA expression was correlated with some clinicopathologic features, including

lymphovascular infiltration and lymph node metastasis, and with poor prognosis (Table 1 and Figure 1). These results suggest that ALDOA is not just highly expressed in cervical adenocarcinoma but might play roles in disease progression, as suggested by the results of cell-based assays.

As shown in Figure 2, knockdown of ALDOA significantly suppressed proliferation, migration, and invasiveness of cervical adenocarcinoma cells, indicating that ALDOA expression contributes to malignant potentials of cervical adenocarcinoma cells. Previous studies showed that ALDOA is highly expressed in malignant neoplasia of various organs and is involved in malignant behaviors of cancer cells.<sup>19-25,38</sup> Cervical adenocarcinoma is now included in the list of malignancies with high ALDOA expression, and our results indicate

that ALDOA is a potential biomarker and a potential therapeutic target of cervical adenocarcinoma.

Interestingly, our cell biological experiments revealed that overexpression of ALDOA caused drastic morphological changes in cervical adenocarcinoma cells (Figure 5). The observed cuboidal-to-spindle morphological change and loss of microvilli are typical features of EMT cells.<sup>39</sup> The ALDOA-induced morphological changes were accompanied by suppression of the expression of adherens and tight junction proteins including E-cadherin,  $\beta$ -catenin, and JAM-A (Figure 6), which has also been shown to occur during the EMT process.<sup>40</sup> As described above, ALDOA has been shown to be highly expressed in malignant neoplasia of various organs and to be involved in malignant behaviors of cancer cells; however, the underlying molecular mechanisms have not been fully elucidated.<sup>41,42</sup> We found that overexpression of ALDOA induced the expression of HIF-1 $\alpha$ , which is known to modulate EMT through regulation of EMT-related transcription factors including Twist, Snail, Slug, Smad interacting protein 1 (Sip1), and zinc finger E-box-binding homeobox 1 (ZEB1).<sup>43-46</sup> In this study, we also confirmed that some of the transcription factors were increased by ALDOA overexpression (Figure 6) and they are probably responsible for downregulation of adherens and tight junction proteins (Figure 6). Our results clearly showed that overexpression of ALDOA could induce EMT in cervical adenocarcinoma cells. ALDOA-induced EMT may explain the positive correlations of high ALDOA expression with lymph node metastasis and lymphovascular infiltration in surgical specimens.

Of note, we observed that a hypoxic condition, a representative factor activating HIF-1 $\alpha$ , induced ALDOA expression in cervical adenocarcinoma cells (Figure 3). Together with the results showing that ALDOA induced HIF-1 $\alpha$  expression (Figure 6), we propose that ALDOA and HIF-1 $\alpha$  form a positive feedback loop to promote EMT-related tumor progression in cervical adenocarcinoma (Figure 6). In this study, we also found that overexpression of ALDOA induced the expression of CD44 and ALDH1 (Figure 6). A future study should be carried out to determine whether ALDOA overexpression leads to the emergence of a stem cell-like property through induction of EMT as induction of CD44 and ALDH1 might reflect the acquisition of cancer stemness.<sup>47</sup>

Metabolic reprogramming, a concept that has emerged in the past few decades, refers to cellular adaptation mechanisms by comprehensive alteration in lipid, glutamine, and sugar metabolism in cancer cells.<sup>45,48</sup> Aldolase A is one of the major glycolytic enzymes, and overexpression of ALDOA could play crucial roles in metabolic reprogramming of cervical adenocarcinoma because the process of metabolic reprogramming is frequently triggered by increased expression and/or activity of metabolism-related enzymes.<sup>49</sup> How metabolic pathways are influenced by ALDOA overexpression in cervical adenocarcinoma should be examined in future studies. The results of such studies would contribute to the establishment of therapeutic strategies for high ALDOA expression-related cancers, including cervical adenocarcinoma.

In conclusion, ALDOA is overexpressed in cervical adenocarcinoma and contributes to malignant potentials of tumor cells through modulation of HIF-1 $\alpha$  signaling. The feedback loop between ALDOA

and HIF-1 $\alpha$  could become a therapeutic target to improve the prognosis of this malignancy.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## REFERENCES

- Bray F, Carstensen B, Møller H, et al. Incidence trends of adenocarcinoma of the cervix in 13 European countries. *Cancer Epidemiol Biomarkers Prevention*. 2005;14(9):2191-2199.
- Bulk S, Visser O, Rozendaal L, et al. Cervical cancer in the Netherlands 1989-1998: decrease of squamous cell carcinoma in older women, increase of adenocarcinoma in younger women. *Int J Cancer*. 2005;113(6):1005-1009.
- Gien LT, Beauchemin MC, Thomas G. Adenocarcinoma: A unique cervical cancer. *Gynecol Oncol*. 2010;116:140-146.
- Sasieni P, Adams J. Changing rates of adenocarcinoma and adenosquamous carcinoma of the cervix in England. *Lancet*. 2001;357:1490-1493.
- Smith HO, Tiffany MF, Qualls CR, et al. The rising incidence of adenocarcinoma relative to squamous cell carcinoma of the uterine cervix in the United States-A 24-year population-based study. *Gynecol Oncol*. 2000;78:97-105.
- Vizcaino AP, Moreno V, Bosch FX, et al. International trends in the incidence of cervical cancer: I. Adenocarcinoma and adenosquamous cell carcinomas. *Int J Cancer*. 1998;75:536-545.
- Ino Y, Akimoto T, Takasawa A, et al. Elevated expression of G protein-coupled receptor 30 (GPR30) is associated with poor prognosis in patients with uterine cervical adenocarcinoma. *Histol Histopathol*. 2020;35:18157.
- Lee J, Kim YT, Kim S, et al. Prognosis of cervical cancer in the era of concurrent chemoradiation from National database in Korea: a comparison between squamous cell carcinoma adenocarcinoma (Ci). *PLoS One*. 2015;10(12):e0144887.
- Mabuchi S, Okazawa M, Matsuo K, et al. Impact of histological subtype on survival of patients with surgically-treated stage IA2-IIIB cervical cancer: adenocarcinoma versus squamous cell carcinoma. *Gynecol Oncol*. 2012;127(1):114-120.
- Nakanishi T, Ishikawa H, Suzuki Y, et al. A comparison of prognoses of pathologic stage Ib adenocarcinoma and squamous cell carcinoma of the uterine cervix. *Gynecol Oncol*. 2000;79(2):289-293.
- Wright TC, Ferenczy A, Kurman RJ. Carcinoma and other tumors of the cervix. In: Kurman RJ, ed. *Blaustein's Pathology of The Female Genital Tract*, 5th edn. New York: Springer-Verlag; 2002:325-382.
- Yokoi E, Mabuchi S, Takahashi R, et al. Impact of histological subtype on survival in patients with locally advanced cervical cancer that were treated with definitive radiotherapy: adenocarcinoma/adenosquamous carcinoma versus squamous cell carcinoma. *J Gynecol Oncol*. 2017;28(2):e19.

13. Akimoto T, Takasawa A, Murata M, et al. Analysis of the expression and localization of tight junction transmembrane proteins, claudin-1, -4, -7, occludin and JAM-A, in human cervical adenocarcinoma. *Histol Histopathol.* 2016;31:921-931.
14. Akimoto T, Takasawa A, Takasawa K, et al. Estrogen/GPR30 signaling contributes to the malignant potentials of ER-negative cervical adenocarcinoma via regulation of claudin-1 expression. *Neoplasia.* 2018;20(10):1083-1093.
15. McCluggage WG. New developments in endocervical glandular lesions. *Histopathol.* 2013;62(1):138-160.
16. Soonthornthum T, Arias-Pulido H, Joste N, et al. Epidermal growth factor receptor as a biomarker for cervical cancer. *Ann Oncol.* 2011;22(10):2166-2178.
17. Mukai T, Joh K, Arai Y, et al. Tissue-specific expression of rat aldolase A mRNAs. Three molecular species differing only in the 5'-terminal sequences. *J Biol Chem.* 1986;261(7):3347-3354.
18. Asaka M, Kimura T, Meguro T, et al. Alteration of aldolase isozymes in serum and tissues of patients with cancer and other diseases. *J Clin Lab Anal.* 1994;8(3):144-148.
19. Du S, Guan Z, Hao L, et al. Fructose-bisphosphate aldolase a is a potential metastasis-associated marker of lung squamous cell carcinoma and promotes lung cell tumorigenesis and migration. *PLoS One.* 2014;9(1):e85804.
20. Poschmann G, Sitek B, Sipos B, et al. Identification of proteomic differences between squamous cell carcinoma of the lung and bronchial epithelium. *Mol Cell Proteomics.* 2009;8(5):1105-1116.
21. Chaerkady R, Harsha HC, Nalli A, et al. A Quantitative proteomic approach for identification of potential biomarkers in hepatocellular carcinoma. *J Proteome Res.* 2008;7(10):4289-4298.
22. Yamamoto T, Kudo M, Peng W-X, et al. Identification of aldolase A as a potential diagnostic biomarker for colorectal cancer based on proteomic analysis using formalin-fixed paraffin-embedded tissue. *Tumor Biol.* 2016;37(10):13595-13606.
23. Long F, Cai X, Luo W, et al. Role of aldolase A in osteosarcoma progression and metastasis: In vitro and in vivo evidence. *Oncol Rep.* 2014;32(5):2031-2037.
24. Chen X, Yang T-T, Zhou Y, et al. Proteomic profiling of osteosarcoma cells identifies ALDOA and SULT1A3 as negative survival markers of human osteosarcoma. *Mol Carcinogen.* 2014;53(2):138-144.
25. Ji S, Zhang BO, Liu J, et al. ALDOA functions as an oncogene in the highly metastatic pancreatic cancer. *Cancer Lett.* 2016;374(1):127-135.
26. Chang Y-C, Yang Y-C, Tien C-P, et al. Roles of aldolase family genes in human cancers and diseases. *Trends Endocrinol Metabol.* 2018;29(8):549-559.
27. Kawai K, Uemura M, Munakata K, et al. Fructose-bisphosphate aldolase A is a key regulator of hypoxic adaptation in colorectal cancer cells and involved in treatment resistance and poor prognosis. *International J Oncol.* 2017;50(2):525-534.
28. Zhang F, Lin J-D, Zuo X-Y, et al. Elevated transcriptional levels of aldolase A (ALDOA) associates with cell cycle-related genes in patients with NSCLC and several solid tumors. *BioData Mining.* 2017;10:1-20.
29. Takasawa A, Murata M, Takasawa K, et al. Nuclear localization of tricellulin promotes the oncogenic property of pancreatic cancer. *Sci Rep.* 2016;6:33582.
30. Hayakawa O, Kudo R, Koizumi M, et al. Establishment of a human adenocarcinoma cell line, CAC-1. *Sapporo Med J.* 1988;57:603-611.
31. Zheng P-S, Iwasaka T, Ouchida M, et al. Growth suppression of a cervical cancer cell line (TMCC-1) by the human wild-type p53 gene. *Gynecol Oncol.* 1996;60:245-250.
32. Aoyama T, Takasawa A, Takasawa K, et al. Identification of coiled-coil domain-containing protein 180 and leucine-rich repeat-containing protein 4 as potential immunohistochemical markers for liposarcoma based on proteomic analysis using formalin-fixed, paraffin-embedded tissue. *Am J Pathol.* 2019;189(5):1015-1028.
33. Magara K, Takasawa A, Osanai M, et al. Elevated expression of JAM-A promotes neoplastic properties of lung adenocarcinoma. *Cancer Sci.* 2017;108(11):2306-2314.
34. Takasawa K, Takasawa A, Osanai M, et al. Claudin-18 coupled with EGFR/ERK signaling contributes to the malignant potentials of bile duct cancer. *Cancer Lett.* 2017;403:66-73.
35. Masuda T, Tomita M, Ishihama Y. Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. *J Proteome Res.* 2008;7(2):731-740.
36. Kanda Y. Investigation of the freely available easy-to-use software 'EZ' for medical statistics. *Bone Marrow Transplant.* 2013;48:452-458.
37. Greijer AE, van der Groep P, Kemming D, et al. Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). *J Pathol.* 2005;206(3):291-304.
38. Chang Y-C, Chiou J, Yang Y-F, et al. Therapeutic targeting of aldolase A interactions inhibits lung cancer metastasis and prolongs survival. *Cancer Res.* 2019;79(18):4754-4766.
39. Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *BioEssays.* 2001;23(10):912-923.
40. Lan M, Kojima T, Murata M, et al. Phosphorylation of ezrin enhances microvillus length via a p38 MAP-kinase pathway in an immortalized mouse hepatic cell line. *Exp Cell Res.* 2006;312(2):111-120.
41. Jiang Z, Wang X, Li J, et al. Aldolase A as a prognostic factor and mediator of progression via inducing epithelial-mesenchymal transition in gastric cancer. *J Cell Mol Med.* 2018;22(9):4377-4386.
42. Ye F, Chen Y, Xia LU, et al. Aldolase A overexpression is associated with poor prognosis and promotes tumor progression by the epithelial-mesenchymal transition in colon cancer. *Biochem Biophys Res Commun.* 2018;497(2):639-645.
43. Bolos V, Peinado H, Perez-Moreno MA, et al. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. *J Cell Sci.* 2016;129(6):1283.
44. Jiang J, Tang YL, Liang XH. EMT: A new vision of hypoxia promoting cancer progression. *Cancer Biol Ther.* 2011;11(8):714-723.
45. Sciacovelli M, Frezza C. Metabolic reprogramming and epithelial-to-mesenchymal transition in cancer. *FEBS J.* 2017;284(19):3132-3144.
46. Yeo CD, Kang N, Choi SY, et al. The role of hypoxia on the acquisition of epithelial-mesenchymal transition and cancer stemness: a possible link to epigenetic regulation. *Korean J Int Med.* 2017;32(4):589-599.
47. Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene.* 2010;29(34):4741-4751.
48. Yoshida GJ. Metabolic reprogramming: the emerging concept and associated therapeutic strategies. *J Exp Clin Cancer Res.* 2015;34:111.
49. Grandjean G, de Jong PR, James BP, et al. Definition of a novel feed-forward mechanism for glycolysis-HIF1 $\alpha$  signaling in hypoxic tumors highlights aldolase A as a therapeutic target. *Cancer Res.* 2016;76(14):4259-4269.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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