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Regular Article

Fructose-bisphosphate Aldolase C Expression is Associated with Poor Prognosis and Stemness in Gastric Cancer

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Gastric cancer (GC) is the third leading cause of cancer-related deaths in Japan, underscoring the urgent need for deeper insights into its pathogenesis. Spheroids provide a more realistic and versatile model for studying cancers and cancer stem cells (CSCs). While fructose-bisphosphate aldolase C (ALDOC) has been identified in colorectal cancer spheroids, its role in GC has remained largely unexplored. This study aimed to elucidate the role of ALDOC in GC by performing single-cell and functional analyses of GC spheroids and cell lines, along with immunohistochemistry of 127 GC samples to assess its correlation with CSC markers. Our single-cell analysis revealed upregulation of ALDOC in spheroids, with pseudotime analysis indicating that ALDOC-expressing cells were predominantly undifferentiated and co-expressed LGR5 and CD44. Further investigation into cell-cell interactions suggested that the stem cell state may be maintained by WNT, BMP, and EGF signaling. Functional assays demonstrated that ALDOC knockdown led to a marked reduction in the growth, invasiveness, and spheroid colony formation capacity of GC cell lines. Clinically, ALDOC was detected in the cytoplasm of 56.7% (72/127) of GC cases, and high ALDOC expression was significantly associated with poor overall survival ($p < 0.01$), and was an independent prognostic factor. Moreover, a significant association between ALDOC and CD44 expression in GC ($p = 0.031$). Conclusively, our findings identify ALDOC as a crucial prognostic marker and provide new insights into GC pathogenesis.

Key words: ALDOC, cancer stem cell, gastric cancer, pathology, spheroid culture

I. Introduction

Gastric cancer (GC), the third most common cause of cancer-related deaths in Japan [38], poses a significant clinical challenge, especially in its advanced stages [32]. Notably, GC development is further complicated by the crucial role of cancer stem cells (CSCs), which contribute to tumor growth, spread, and treatment resistance [4, 35]. GC stem cells are characterized by the expression of CD44 [36] and other molecules [27]. Research findings indicate that spheroid culture is a useful method for analyzing CSCs [35]. Previously, several potential CSC markers have been

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identified using spheroid culture [23, 24]. Besides this, the spheroid colony formation assay has become a widely used method for observing stemness [3, 30]. Although single-cell analysis techniques have emerged in recent years [37] and the innovation has been remarkable, single-cell analysis has not been performed on spheroids. Currently, therapeutic target molecules for GC are limited to CLDN18, PD-L1, and HER2 [29]. Single-cell level analysis is required in GC, and it is hoped that, if possible, candidate therapeutic targets can be found.

Fructose-bisphosphate aldolase C (ALDOC), also known as zebrin II [1], belongs to the aldolase family, including ALDOA and ALDOB, which are enzymes involved in glycolysis [5]. ALDOC is abundantly localized in the central nervous system [21] and is especially expressed in Purkinje cells in the cerebellum [8] and in Schwann cells [39]. Additionally, ALDOC has been identified as a marker of folliculostellate cells in the anterior pituitary gland of rodents [9]. ALDOC has been reported to be ectopically expressed in tumor cells [5] and highly upregulated in breast cancer [28], glioblastoma [15], and non-small cell lung cancer cells [31], contributing to tumor malignancy. A previous study showed that ALDOC contributes to spheroid formation by colon cancer cell lines and that its high expression worsens the prognosis of colorectal cancer [19]. However, the role of ALDOC in GC remains unexplored, although a few studies have shown that it is significantly associated with immune infiltration in GC, regulates macrophage differentiation, promotes GC progression [6], and is expressed as specialized splicing variants [11]. Notably, there are no reports on the association between ALDOC and cancer stemness.

Therefore, in this study, we aimed to investigate the role of ALDOC in GC using single-cell and functional analyses of GC spheroids and cell lines. Specifically, we compared the transcriptomes of spheroids and cell lines in normal cultures using single-cell analysis. Additionally, we performed functional analysis of ALDOCs using cell lines. Immunohistochemical analysis was performed on 127 resected GC specimens to elucidate the association between ALDOC expression and the clinicopathological characteristics of patients.

II. Materials and Methods

Single-cell RNA sequencing

Single-cell RNA sequencing analysis was performed on MKN-45, a human-derived GC cell line, under 2D (conventional culture conditions) and 3D (spheroid culture conditions) culture conditions. Library preparation was performed at Natural Science Center for Basic Research and Development in Hiroshima University using Chromium Next GEM Single Cell 3' LT Reagent Kits (v3.1 Dual Index, PN-1000215, 10× Genomics, Pleasanton, CA, USA). Libraries were sequenced using an MGI DNBseq-G400 (MGI Tech Co., Shenzhen, China). The Space Ranger pipeline v2022.0705.1 (10× Genomics, Pleasanton, CA, USA) and the GRCh38-2020- A reference set was used to process the FASTQ files.

Gene spot matrices were analyzed using the Seurat (version 5.1.0) of R package (version 4.4.1) [33]. For each data sample, spots were filtered to obtain a minimum detected gene count of 100 genes. Normalization across spots was performed using the SCTransform package (version 0.4.1) [10]. Dimensionality reduction and clustering were performed using independent component analysis. Computational analysis of compartment embedding trajectories was performed using the Monocle 3 (version 1.3.7) algorithm according to the method described by Qiu *et al.* [26] CellChat (version 1.6.1) was used to analyze cell-cell interactions. [14]

Tissue samples

In this study, 127 primary GC samples were collected from patients who underwent curative resection between 2012 and 2015 at the Kure Medical Center and Chugoku Cancer Center (Kure, Hiroshima, Japan). One representative tumor block from each specimen was assessed using immunohistochemistry (IHC). All GC tumor stages were based on the Japanese Classification of gastric cancer [13] and the tumor, node, and metastasis classifications of the Union for International Cancer Control.

Data availability

The raw scRNA-seq data obtained in this study were deposited in the DNA Data Bank of Japan (DDBJ) under the accession code PRJDB18575 (DRR585583–DRR585584).

Cell lines

The human GC cell lines, MKN-1, MKN-7, MKN-45, and MKN-74, were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All cell lines were maintained in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd.) containing 10% fetal bovine serum (Corning) at 37°C in a humidified atmosphere with 5% CO_2.

RNA interference (RNAi)

Small interfering RNA (siRNA) targeting ALDOC and negative control oligonucleotides were purchased from Invitrogen (#1299003, Carlsbad, CA, USA). Three independent ALDOC-specific siRNA oligonucleotide sequences were used. Briefly, GC cell lines were transfected with the siRNAs using Lipofectamine RNAiMAX (#13778075, Invitrogen, Waltham, Massachusetts, USA). After 48 hr of transfection, GC cells were analyzed.

Cell growth assay

Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess cell growth. GC cells were seeded in 96-well plates at a density of 3000 cells/well, and cell growth was monitored after 1, 2, and 4

days. Four separate MTT experiments were performed and the mean ± standard deviation (SD) was calculated.

Invasion assay

A modified Boyden chamber assay was performed to examine cell invasiveness using the Cell Invasion Assay Kit (ECM550; Merck KGaA, Darmstadt, Germany). Briefly, cells were seeded at 1×10^5 cells in RPMI-1640 without serum in the upper chamber of a culture insert (8-μm pore size). A medium containing 10% serum was added to the bottom chamber. After incubating at 37°C for 48 hr, the lower surface of the insert was stained with cell stain to assess the number of cells, as previously described [19].

Cell migration assay

Wound healing assays were performed to monitor cell migration using the ibidi 2 Well Culture-Insert (ibidi GmbH). Briefly, GC cells were suspended at a concentration of 1×10^6 cells/mL in RPMI-1640 medium, and 70 μL of cells were added to each well. After 24 hr, the inserts were gently removed and the cells were cultured in 10% serum RPMI-1640 medium. Images were obtained at appropriate time points using phase-contrast microscopy.

Spheroid colony formation assay

For spheroid generation, 2,000 cells were seeded in 6-well ultra-low attachment plates (#3471; Corning, Arizona, United States). The cells were grown in mTeSR medium (#85850; STEMCELL Technologies Inc., Vancouver, BC, Canada). Spheroid number and size were determined using a microscope.

Immunohistochemistry (IHC)

For IHC, representative formalin-fixed paraffin-embedded (FFPE) blocks were cut into small sections (4 μm), deparaffinized, and rehydrated. Immunohistochemical analysis was performed using a Dako EnVision+ Peroxidase Detection System (#K4003, Dako Cytomation, Carpinteria, CA, USA). Briefly, FFPE tissue sections were treated with sodium citrate buffer (pH 6.0) for 30 min in a microwave for antigen retrieval, followed by treatment with 3% H_2O_2-methanol for 10 min to block peroxidase activity. Thereafter, the sections were incubated with rabbit polyclonal anti-ALDOC antibody (1:400, AV48273; Sigma-Aldrich) at room temperature for 1 hr, followed by incubation with EnVision+ peroxidase-conjugated anti-rabbit secondary antibody for 1 hr. For the color reaction, the sections were incubated with the Dako Liquid DAB+ Substrate Chromogen System (#K3468, Santa Clara, CA, USA) for 5 min. Sections were counterstained with 0.1% hematoxylin. ALDOC expression was evaluated as positive or negative for all slides. Sample was considered positive for ALDOC when >10% of the tumor cell cytoplasm were stained, as in a previous study [19]. Notably, the ALDOC antibodies used in this study have proven effective in previous studies, including in siRNA knockdown experiments [19]. Additionally, immunostaining for CD44 was performed using a mouse monoclonal anti-CD44 antibody (1:100, clone DF1485; Novocastra, Newcastle upon Tyne, UK). Other methods, including antigen retrieval, were the same as those used for the ALDOC. Finally, the immunoreactivity of each specimen was independently assessed by two surgical pathologists (A.I. and N.K.).

Statistical analysis

Correlations between clinicopathological parameters and ALDOC expression were analyzed using the Fisher's exact test. Differences between the survival curves were analyzed using log-rank tests. Univariate and multivariate Cox regression analyses were performed to evaluate the association between clinical covariates and overall survival. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using Cox proportional hazard models. Statistical significance was set at $p < 0.05$.

III. Results

ALDOC expression levels based on single cell analysis

In this study, we performed single-cell RNA sequencing of a human-derived GC cell line, MKN-45, and compared the normal 2D culture conditions with specialized 3D spheroid culture conditions. An integrated analysis of these cells yielded four cell populations on demention reduction using UMAP, among which 2068 cells were analyzed (Fig. 1A). ALDOC expression was determined by constructing a FeaturePlot by UMAP (Fig. 1B). ALDOC expression was mainly observed in clusters 1 and 3, and trajectory analysis showed that these clusters had low pseudotime values by monocle3 (Fig. 1C). ALDOC expression levels are depicted by FeaturePlots in 2D (1067 cells) and 3D (1001 cells) states (Fig. 1D). Then, focusing on the 3D state, the following analysis was performed. We compared the expression of LGR5, known stem cell markers, or CD44, a known CSC marker, in the integrated cell population using FeaturePlot (Fig. 1E). Notably, ALDOC was highly expressed in the 3D spheroid state, and its expression was in closely related to that of LGR5 and CD44. From here, we further examined the interactions between these cells. Significant ligand and receptor expression among each cluster was comprehensively examined with a heatmap (Supplementary Fig. S1A). Among them, we focused on WNT, BMP, and EGF, which are related to stem cell maintenance, and showed a heatmap of recipients and sources (sender) of each cluster (Supplementary Fig. S1B). Then, we examined which molecular pairs were the focus of those interactions (Supplementary Fig. S1C). They are shown visually in the chord diagram (Fig. 1F). Generally similar results were obtained with both 2D state and 3D state cells in the heatmap (Supplementary Fig. S2A), chord diagram (Supplementary Fig. S2B), focused heatmap (Supplementary Fig. S2C), and ligand-receptor relationship diagrams (Supplementary Fig. S2D). These results suggest that

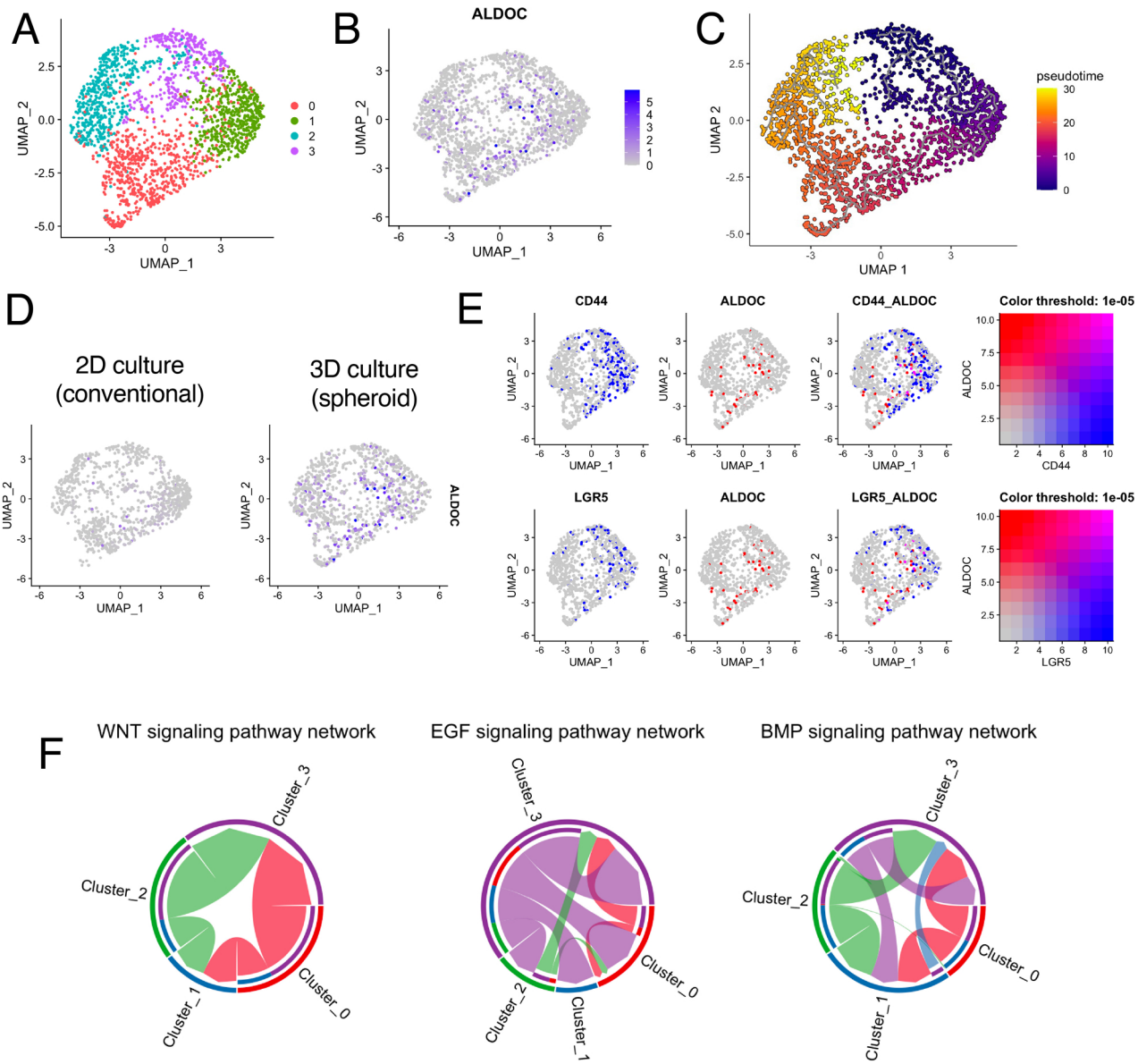

Fig. 1. Single-cell analysis of MKN-45 gastric cancer cells. (**A**) UMAP dimensional reduction analysis of 2068 cells from MKN-45, cultured under normal 2D and specialized 3D spheroid conditions, identified four distinct cell populations. (**B**) FeaturePlot displaying fructose-bisphosphate aldolase C (ALDOC) expression across the identified cell populations in the UMAP. ALDOC expression is predominantly observed in clusters 1 and 3. (**C**) Trajectory analysis by Monocle3 revealed that clusters 1 and 3, where ALDOC is expressed, are associated with low pseudotime values. (**D**) Comparison of ALDOC expression in 2D (1067 cells) and 3D (1001 cells) culture conditions, visualized via FeaturePlots, shows higher ALDOC expression in the 3D spheroid state. (**E**) FeaturePlot analysis of LGR5 and CD44 expression indicates a close association of ALDOC expression with these known stem cell and CSC markers. (**F**) Chord diagram illustrating significant ligand-receptor interactions among cell clusters, focusing on WNT, BMP, and EGF signaling pathways.

ALDOC expression is found in CSC-like clusters in which CD44 and LGR5 are expressed and that these clusters are maintained by autocrine WNT, EGF, and BMP signaling.

ALDOC knockdown inhibits GC cell proliferation, invasion, and spheroid colony-formation capacity

GC cells were transfected with ALDOC-specific siRNAs to examined the significance of ALDOC in GC. Western blot analysis showed that ALDOC was expressed in all MKN cells (Fig. 2A). Additionally, siRNA-mediated ALDOC knockdown was confirmed in MKN-45 (Fig. 2B) and MKN-1 cells using western blotting (Fig. 2C). Cell proliferation and invasion assays showed that ALDOC knockdown significantly decreased the growth and invasive ability of the GC cell lines MKN-45 and MKN-1 compared with that in the control siRNA-transfected group (Fig. 2D–G). Additionally, we examined the association between ALDOC expression and spheroid colony formation by GC

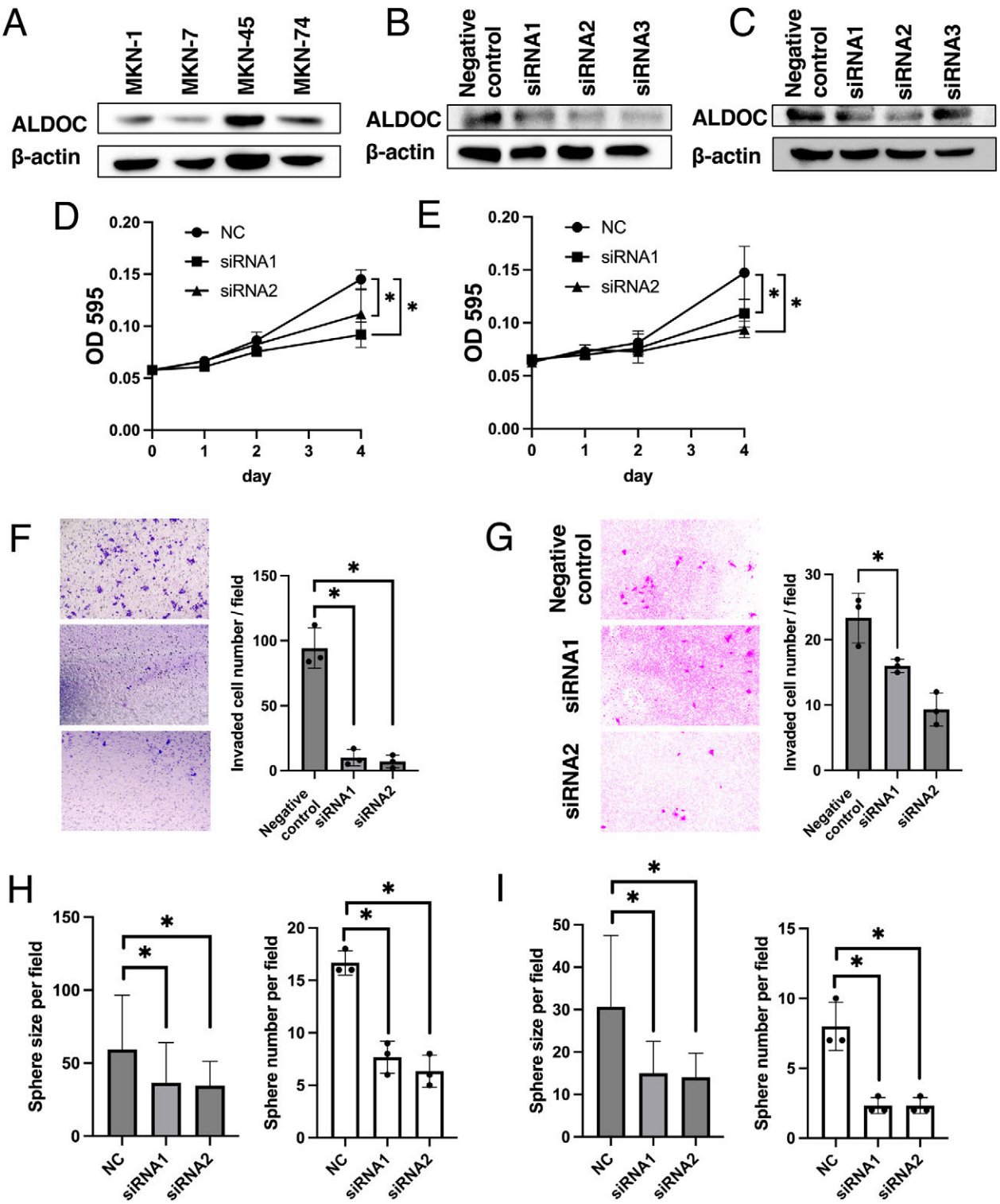

Fig. 2. Effects of fructose-bisphosphate aldolase C (ALDOC) inhibition on gastric cancer (GC) cells. (**A**) Western blot analysis of ALDOC in four GC cell lines. (**B** and **C**) Western blot analysis of ALDOC in MKN-45 (**B**) and MKN-1 (**C**) cells transfected with negative control or ALDOC small interfering RNA (siRNA). (**D** and **E**) Effect of ALDOC knockdown on the growth of MKN-45 (**D**) and MKN-1 (**E**) cells transfected with the negative control or ALDOC siRNA. (**F** and **G**) Images of the invasion assay in MKN-45 (**F**) and MKN-1 (**G**) cells transfected with ALDOC siRNA or negative control siRNA and quantification of the average number of invaded cells. (**H** and **I**) Number and size of spheroids formed by MKN-45 (**H**) and MKN-1 (**I**) cell lines transfected with negative control or ALDOC siRNA.

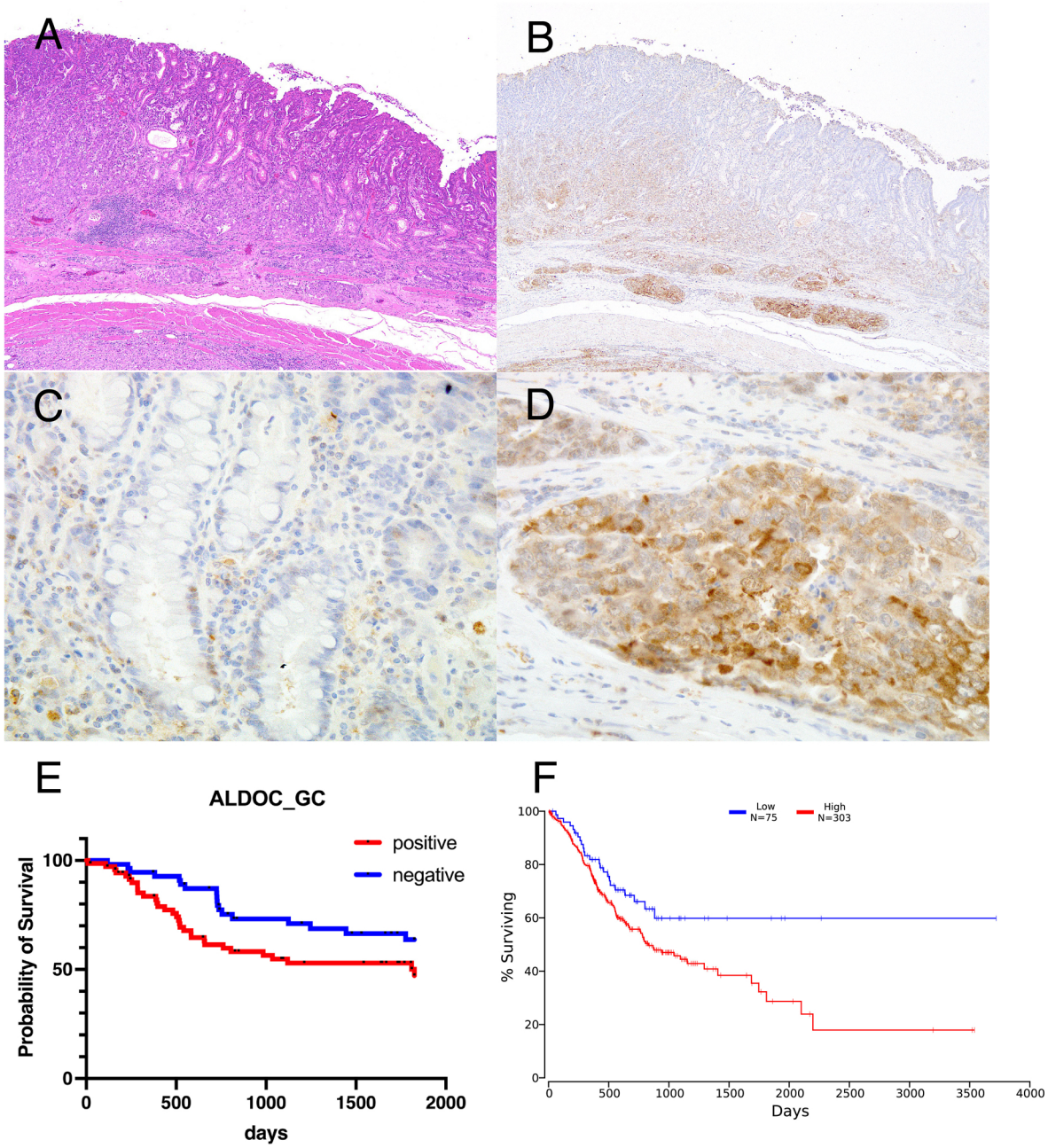

Fig. 3. Immunohistochemical analysis of fructose-bisphosphate aldolase C (ALDOC) expression in gastric cancer (GC) tissue samples. (**A**) Corresponding Hematoxylin-Eosin stain image. (**B**–**D**) Representative images of ALDOC in the non-neoplastic gastric mucosa (**C**) and GC (**B** and **D**). Original magnification: (**A** and **B**) 100× and (**C** and **D**) 400×. (**E**) Overall survival probability of 127 patients with GC. (**F**) Overall survival probability in The Cancer Genome Atlas GC dataset.

cell lines. Then, we performed spheroid colony formation assay to see the effect on stemness. The siRNA-mediated ALDOC knockdown significantly increased the number and size of spheroids formed by MKN-45 and MKN-1 cells (Fig. 2H, I). These results suggest that ALDOC expression may be associated with GC progression and malignancy.

ALDOC is upregulated in GC cells and is associated with poor prognosis

To confirm the results of our cell analysis, we performed immunohistochemical analysis of 127 surgical samples. The Hematoxylin-Eosin (HE) staining corresponding to the representative immunostaining image shown later is shown in Fig. 3A. Immunohistochemical assay indicated sparse staining for ALDOC in GC tissue, whereas the non-

Table 1. *Relationship between ALDOC expression and clinicopathologic characteristics in the 127 gastric cancer cases*

		ALDOC expression		*p* Value
		Positive (%)	Negative	
Location				0.6449
	Upper	15 (63)	9	
	Middle	36 (58)	26	
	Lower	21 (51)	20	
Sex				0.8416
	Male	53 (58)	39	
	Female	19 (54)	16	
pStage				0.0969
	Stage I/II	21 (47)	24	
	Stage III/IV	51 (62)	31	
pT stage				0.0498
	pT1/2	45 (51)	44	
	pT3/4	27 (71)	11	
pN stage				0.5691
	pN0/1	22 (52)	20	
	pN2/3	50 (59)	35	
pM stage				0.5510
	pM0	64 (56)	51	
	pM1	8 (67)	4	
Venous invasion (V)				0.6711
	V0/1a	55 (56)	44	
	V1b/1c	17 (61)	11	
Lymphatic invasion (Ly)				0.3699
	Ly0/1a	35 (52)	32	
	Ly1b/1c	37 (62)	23	
Histology				0.0580
	Intestinal	29 (69)	13	
	Diffuse	43 (51)	42	

neoplastic gastric mucosa was negative or weakly positive (Fig. 3B). Although the intestinal metaplasia was negative for ALDOC, a small number of positive cells were observed in the surrounding lymphocytes (Fig. 3C). In the strongly positive GC areas, ALDOC was detected mainly in the cytoplasm (Fig. 3D). Samples were considered ALDOC-positive when ALDOC expression was detected in more than 10% of tumor cells. In total, 72 (56.7%) patients with GC were positive for ALDOC. Additionally, we examined the relationship between ALDOC expression and the clinicopathological characteristics of the patients (Table 1). Importantly, ALDOC expression was significantly correlated pT stage ($p = 0.0498$). In contrast, ALDOC expression was not significantly correlated with other factors.

Correlation between ALDOC expression and survival in patients with GC

In this study, we examined the correlation between ALDOC and survival in patients with GC. Patients with ALDOC-positive GC showed a significantly worse ($p < 0.01$) OS than those with ALDOC-negative GC (Fig. 3E). To confirm this result, we used the public TCGA dataset. Analysis of TCGA data in the Oncolnc dataset (http://www.oncolnc.org/) revealed that high ALDOC expression was significantly associated ($p = 0.03$) with poor prognosis in patients with GC (Fig. 3F). Univariate and multivariate Cox proportional hazards analyses were performed to evaluate the potential use of ALDOC expression as a prognostic marker (Table 2). Univariate analysis showed that pT stage (HR, 2.960; 95% CI, 1.700–5.153; $p < 0.01$), pN

Table 2. *Univariate and multivariate Cox regression analyses of ALDOC expression and survival of gastric cancer patients*

Features		Univariate analysis		Multivariate analysis	
		HR (95%CI)	*p* Value	HR (95%CI)	*p* Value
Sex			0.460		
	Female	1 (ref.)			
	Male	1.251 (0.691–2.266)			
Venous invasion (V)			0.132		
	V0	1 (ref.)			
	V1	1.570 (0.873–2.821)			
Lymphatic invasion (Ly)			0.167		
	Ly0	1 (ref.)			
	Ly1	1.485 (0.847–2.603)			
pT stage			<0.01		0.046
	pT1/2	1 (ref.)		1 (ref.)	
	pT3/4	2.960 (1.700–5.153)		1.821 (1.012–3.278)	
pN stage			<0.01		<0.01
	pN0/1	1 (ref.)		1 (ref.)	
	pN2/3	6.314 (2.271–17.56)		4.316 (1.521–12.25)	
pM stage			<0.01		0.218
	pM0	1 (ref.)		1 (ref.)	
	pM1	2.815 (1.451–5.458)		1.566 (0.767–3.194)	
Histology			0.081		
	Intestinal	1 (ref.)			
	Diffuse	1.760 (0.932–3.320)			
ALDOC expression			<0.01		0.040
	Negative	1 (ref.)		1 (ref.)	
	Positive	2.892 (1.561–5.360)		1.956 (1.030–3.718)	

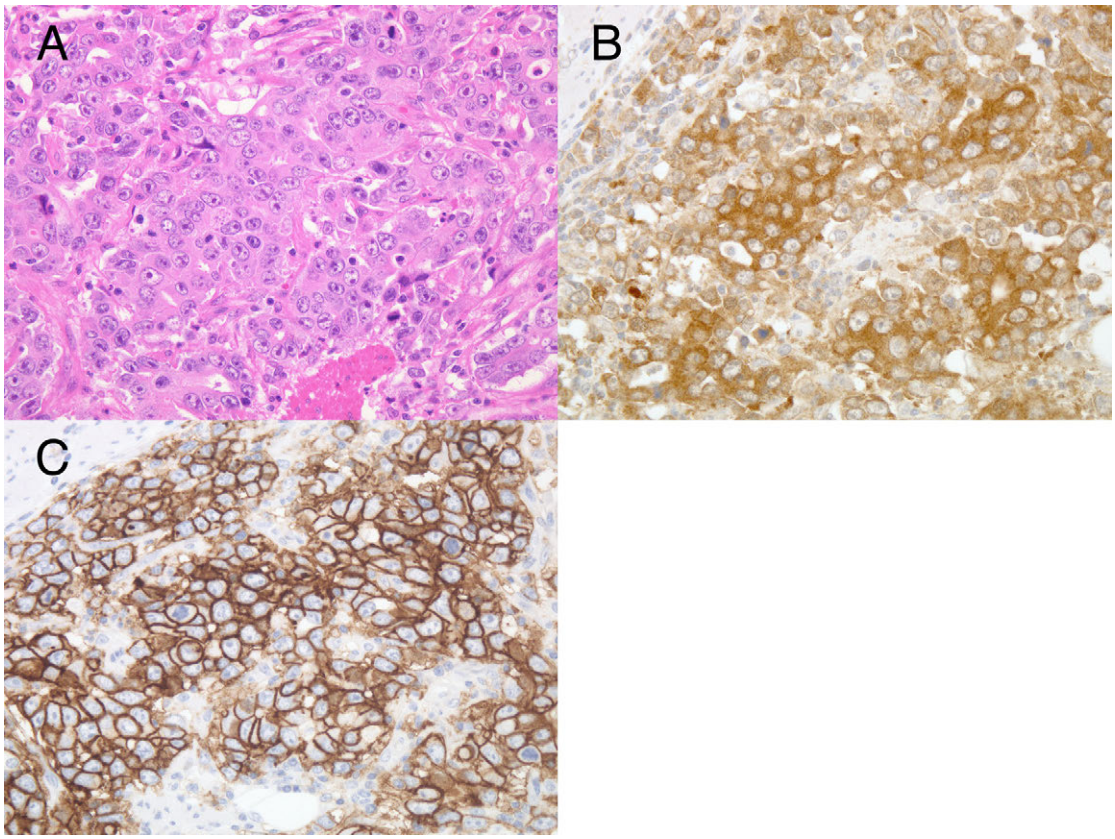

Fig. 4. Immunohistochemical analyses of ALDOC and CD44 expression. (**A**) Corresponding Hematoxylin-Eosin stain image. (**B**) ALDOC. (**C**) CD44. Original magnification: (**A**–**C**) 400×.

stage (HR, 6.314; 95% CI, 2.271–17.56; $p < 0.01$), pM stage (HR, 2.815; 95% CI, 1.451–5.458; $p < 0.01$), and ALDOC expression (HR, 2.892; 95% CI, 1.561–5.360; $p < 0.01$) were associated with poor survival. Additionally, multivariate analysis showed that ALDOC expression (HR, 1.956; 95% CI, 1.030–3.718; $p = 0.040$) was an independent predictor of survival in patients with GC. Collectively, these results suggest that ALDOC is a potential biomarker for identifying patients with a poor prognosis.

Correlation between ALDOC and CD44, a CSC marker

Immunostaining was performed on 127 GC tissue samples to investigate the correlation between ALDOC expression and the CSC marker CD44. The HE staining corresponding to the representative immunostaining image shown later is shown in Fig. 4A. In total, 64 (50%) of the 127 GC cases were CD44-positive. A comparison of the staining patterns showed that ALDOC (Fig. 4B) and CD44 (Fig. 4C) were expressed in the same tumor cells. Additionally, ALDOC-positive GC cases were significantly ($p = 0.031$) found among CD44-positive GC cases (Table 3). Collectively, these results indicate that ALDOC contributes to the associated stemness of CD44 in clinical specimens.

Table 3. *The relationship between ALDOC expression and CD44 in patients with gastric cancer*

		ALDOC expression		*p* Value
		Positive (%)	Negative	
CD44				0.031
	Positive	42 (66)	22	
	Negative	30 (47)	33	

IV. Discussion

ALDOC expression has been showed to be an indicator of poor prognosis in colorectal cancer [19]. Although we have previously performed a single-cell analysis [12], this is the first study to show that ALDOC is involved in the stemness of GC cells. In addition, this is the first attempt to perform single-cell analysis on spheroids of GC cell line.

In this study, ALDOC and CSCs were identified at three levels: single-cell, GC cell lines, and clinical specimens. Although some studies have shown an association between ALDOC expression and spheroid-forming capacity [7], there are no reports on the relationship between spe-

cific CSC markers and ALDOC expression [19]. ALDOC knockdown is speculated to reduce lactate production, thereby reducing spheroid-forming capacity [7, 19] and promoting metabolic reprogramming [31]. In the present study, we identified a CSC marker (CD44) that showed consistent expression level at both the single-cell and tissue levels. CD44 is a type I transmembrane glycoprotein that acts as a receptor for the extracellular matrix component hyaluronic acid [22, 40]. Notably, CD44 is widely known to be a cancer stem cell marker in several cancer types [17, 34]. For example, several genes associated with CD44 have been identified in pancreatic cancer and GC [35, 36]. In examining cell-cell interactions in this study, it is possible that these stem cell-like cell populations maintain their own stemness via WNT, BMP, and EGF, but *in vivo* they receive these supplies from a variety of components other than cancer cells [16, 25]. In this study, single-cell analysis and immunochemical assay of clinical samples showed a correlation between CD44 and ALDOC expression levels. To the best of our knowledge, this is the first study to report a relationship between ALDOC and CD44 expression.

Additionally, analyses of the cell lines and clinical specimens showed that ALDOC contributes to the progression of GC. Research findings indicate that ALDOC expression increases tumor cell proliferation and invasiveness in lung [7, 31], breast [28], and colorectal cancers [19]. Furthermore, correlation analysis between ALDOC expression and the pT stage confirmed increased proliferation and invasiveness of GC cells. In contrast, ALDOC expression improved the prognosis of oral squamous cell carcinoma [18], indicating that the significance of ALDOC expression may vary among cancer types.

Despite the promising findings, this study had some limitations. For example, the cell line used for single-cell analysis was MKN-45, a poorly differentiated GC cell line [20]. Additionally, genetic analysis indicate that MKN-45 is wild-type for p53 and lacks MLH1 mutations [41]. Moreover, although this study has demonstrated a relationship between ALDOC and CD44 under different modalities, we were unable to determine whether the relationship is direct or indirect. Considering that ALDOC is localized in the cytoplasm [19] and CD44 is expressed in the plasma membrane [2], a direct interaction is unlikely, indicating the need for further verification. Moreover, the clinical specimens used in this study were obtained from a single center. Therefore, samples from multiple centers are necessary to confirm whether ALDOC is a potential therapeutic target.

Conclusively, ALDOC is an independent poor prognostic factor in GC and possesses application as a prognostic and CSC marker.

V. Conflicts of Interest

The authors declare that there are no conflicts of interest.

VI. Funding

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VII. Ethical Approval and Informed Consent

This study was approved by the Ethics Committee for Human Genome Research of Hiroshima University (E2001-9923), and conducted in accordance with the guidelines of the Declaration of Helsinki. The opt-out method was used in this retrospective study. Written informed consent was obtained from all patients. An informed consent protocol was used in this study.

VIII. Acknowledgments

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IX. References

1. Ahn, A. H., Dziennis, S., Hawkes, R. and Herrup, K. (1994) The cloning of zebrin II reveals its identity with aldolase C. *Development* 120; 2081–2090.
2. Aruffo, A., Stamenkovic, I., Melnick, M., Underhill, C. B. and Seed, B. (1990) CD44 is the principal cell surface receptor for hyaluronate. *Cell* 61; 1303–1313.
3. Bahmad, H. F., Cheaito, K., Chalhoub, R. M., Hadadeh, O., Monzer, A., Ballout, F., *et al.* (2018) Sphere-Formation Assay: Three-Dimensional in vitro Culturing of Prostate Cancer Stem/ Progenitor Sphere-Forming Cells. *Front. Oncol.* 8; 347.
4. Bessede, E., Dubus, P., Megraud, F. and Varon, C. (2015) Helicobacter pylori infection and stem cells at the origin of gastric cancer. *Oncogene* 34; 2547–2555.
5. Chang, Y. C., Yang, Y. C., Tien, C. P., Yang, C. J. and Hsiao, M. (2018) Roles of Aldolase Family Genes in Human Cancers and Diseases. *Trends Endocrinol. Metab.* 29; 549–559.
6. Chen, L., Zeng, Y., Ren, B., Wang, X., Zhao, F., Du, J., *et al.* (2023) ALDOC regulated the biological function and immune infiltration of gastric cancer cells. *Int. J. Biochem. Cell Biol.* 158; 106407.
7. De Vitis, C., Battaglia, A. M., Pallocca, M., Santamaria, G., Mimmi, M. C., Sacco, A., *et al.* (2023) ALDOC- and ENO2-driven glucose metabolism sustains 3D tumor spheroids growth regardless of nutrient environmental conditions: a multi-omics analysis. *J. Exp. Clin. Cancer Res.* 42; 69.
8. Fujita, H., Aoki, H., Ajioka, I., Yamazaki, M., Abe, M., Oh-Nishi, A., *et al.* (2014) Detailed Expression Pattern of Aldolase C (Aldoc) in the Cerebellum, Retina and Other Areas of the CNS Studied in Aldoc-Venus Knock-In Mice. *PLoS One* 9; e86679.
9. Fujiwara, K., Tsukada, T., Horiguchi, K., Fujiwara, Y., Takemoto, K., Nio-Kobayashi, J., *et al.* (2020) Aldolase C is a novel molecular marker for folliculo-stellate cells in rodent

pituitary. *Cell Tissue Res.* 381; 273–284.
10. Hafemeister, C. and Satija, R. (2019) Normalization and variance stabilization of single-cell RNA-seq data using regularized negative binomial regression. *Genome Biol.* 20; 296.
11. Hatakeyama, K., Ohshima, K., Fukuda, Y., Ogura, S., Terashima, M., Yamaguchi, K., *et al.* (2011) Identification of a novel protein isoform derived from cancer-related splicing variants using combined analysis of transcriptome and proteome. *Proteomics* 11; 2275–2282.
12. Ishikawa, A., Fukui, T., Kido, A., Katsuya, N., Kuraoka, K., Uraoka, N., *et al.* (2024) Discovering cancer stem-like molecule, nuclear factor I X, using spatial transcriptome in gastric cancer. *Cancer Sci.* 115; 3180–3193. doi: 10.1111/cas.16288.
13. Japanese Gastric Cancer Association (2011) Japanese classification of gastric carcinoma: 3rd English edition. *Gastric Cancer* 14; 101–112.
14. Jin, S., Guerrero-Juarez, C. F., Zhang, L., Chang, I., Ramos, R., Kuan, C. H., *et al.* (2021) Inference and analysis of cell-cell communication using CellChat. *Nat. Commun.* 12; 1088.
15. Kathagen-Buhmann, A., Schulte, A., Weller, J., Holz, M., Herold-Mende, C., Glass, R., *et al.* (2016) Glycolysis and the pentose phosphate pathway are differentially associated with the dichotomous regulation of glioblastoma cell migration versus proliferation. *Neuro Oncol.* 18; 1219–1229.
16. Kumar, V., Ramnarayanan, K., Sundar, R., Padmanabhan, N., Srivastava, S., Koiwa, M., *et al.* (2022) Single-Cell Atlas of Lineage States, Tumor Microenvironment, and Subtype-Specific Expression Programs in Gastric Cancer. *Cancer Discov.* 12; 670–691.
17. Lau, W. M., Teng, E., Chong, H. S., Lopez, K. A., Tay, A. Y., Salto-Tellez, M., *et al.* (2014) CD44v8-10 is a cancer-specific marker for gastric cancer stem cells. *Cancer Res.* 74; 2630–2641.
18. Li, Y. J., Huang, T. H., Hsiao, M., Lin, B. R., Cheng, S. J., Yang, C. N., *et al.* (2016) Suppression of fructose-bisphosphate aldolase C expression as a predictor of advanced oral squamous cell carcinoma. *Head Neck* 38 Suppl 1; E1075–E1085.
19. Maruyama, R., Nagaoka, Y., Ishikawa, A., Akabane, S., Fujiki, Y., Taniyama, D., *et al.* (2022) Overexpression of aldolase, fructose-bisphosphate C and its association with spheroid formation in colorectal cancer. *Pathol. Int.* 72; 176–186.
20. Motoyama, T. and Watanabe, H. (1983) Carcinoembryonic antigen production in human gastric cancer cell lines *in vitro* and in nude mice. *Gan* 74; 679–686.
21. Mukai, T., Joh, K., Arai, Y., Yatsuki, H. and Hori, K. (1986) Tissue-specific expression of rat aldolase A mRNAs. Three molecular species differing only in the 5'-terminal sequences. *J. Biol. Chem.* 261; 3347–3354.
22. Naor, D., Sionov, R. V. and Ish-Shalom, D. (1997) CD44: structure, function, and association with the malignant process. *Adv. Cancer Res.* 71; 241–319.
23. Oue, N., Mukai, S., Imai, T., Pham, T. T., Oshima, T., Sentani, K., *et al.* (2016) Induction of KIFC1 expression in gastric cancer spheroids. *Oncol. Rep.* 36; 349–355.
24. Oue, N., Yamamoto, Y., Oshima, T., Asai, R., Ishikawa, A., Uraoka, N., *et al.* (2018) Overexpression of the Transmembrane Protein IQGAP3 Is Associated with Poor Survival of Patients with Gastric Cancer. *Pathobiology* 85; 192–200.
25. Oya, Y., Hayakawa, Y. and Koike, K. (2020) Tumor microenvironment in gastric cancers. *Cancer Sci.* 111; 2696–2707.
26. Qiu, X., Mao, Q., Tang, Y., Wang, L., Chawla, R., Pliner, H. A., *et al.* (2017) Reversed graph embedding resolves complex single-cell trajectories. *Nat. Methods.* 14; 979–982.
27. Quante, M. and Wang, T. C. (2009) Stem cells in gastroenterology and hepatology. *Nat. Rev. Gastroenterol. Hepatol.* 6; 724–737.
28. Reinsborough, C. W., Ipas, H., Abell, N. S., Gouws, E. B., Williams, J. P., Mercado, M., *et al.* (2021) BCDIN3D RNA methyltransferase stimulates Aldolase C expression and glycolysis through let-7 microRNA in breast cancer cells. *Oncogene* 40; 2395–2406.
29. Rocken, C. (2023) Predictive biomarkers in gastric cancer. *J. Cancer Res. Clin. Oncol.* 149; 467–481.
30. Shaheen, S., Ahmed, M., Lorenzi, F. and Nateri, A. S. (2016) Spheroid-Formation (Colonosphere) Assay for in Vitro Assessment and Expansion of Stem Cells in Colon Cancer. *Stem. Cell Rev. Rep.* 12; 492–499.
31. Shang, B., Lu, F., Jiang, S., Xing, M., Mao, X., Yang, G., *et al.* (2023) ALDOC promotes non-small cell lung cancer through affecting MYC-mediated UBE2N transcription and regulating Wnt/β-catenin pathway. *Aging* 15; 9614–9632.
32. Siegel, R. L., Miller, K. D., Wagle, N. S. and Jemal, A. (2023) Cancer statistics, 2023. *CA Cancer J. Clin.* 73; 17–48.
33. Stuart, T., Butler, A., Hoffman, P., Hafemeister, C., Papalexi, E., Mauck, W. M., 3rd, *et al.* (2019) Comprehensive Integration of Single-Cell Data. *Cell* 177; 1888–1902.e21.
34. Sun, M., Zhou, W., Zhang, Y. Y., Wang, D. L. and Wu, X. L. (2013) CD44(+) gastric cancer cells with stemness properties are chemoradioresistant and highly invasive. *Oncol Lett.* 5; 1793–1798.
35. Takaishi, S., Okumura, T. and Wang, T. C. (2008) Gastric cancer stem cells. *J. Clin. Oncol.* 26; 2876–2882.
36. Takaishi, S., Okumura, T., Tu, S., Wang, S. S., Shibata, W., Vigneshwaran, R., *et al.* (2009) Identification of gastric cancer stem cells using the cell surface marker CD44. *Stem Cells* 27; 1006–1020.
37. Tang, F., Barbacioru, C., Wang, Y., Nordman, E., Lee, C., Xu, N., *et al.* (2009) mRNA-Seq whole-transcriptome analysis of a single cell. *Nat. Methods* 6; 377–382.
38. Thuler, L. C. S. (2022) The Epidemiology of Stomach Cancer. In "Gastrointestinal Cancers", ed. by J. A. Morgado-Diaz, Exon Publications, Brisbane (AU).
39. Xu, S., Ao, J., Gu, H., Wang, X., Xie, C., Meng, D., *et al.* (2017) IL-22 Impedes the Proliferation of Schwann cells: Transcriptome Sequencing and Bioinformatics Analysis. *Mol. Neurobiol.* 54; 2395–2405.
40. Yaghobi, Z., Movassaghpour, A., Talebi, M., Abdoli Shadbad, M., Hajiasgharzadeh, K., Pourvahdani, S., *et al.* (2021) The role of CD44 in cancer chemoresistance: A concise review. *Eur. J. Pharmacol.* 903; 174147.
41. Yokozaki, H. (2000) Molecular characteristics of eight gastric cancer cell lines established in Japan. *Pathol. Int.* 50; 767–777.