Overexpression of aldolase, fructose-bisphosphate C and its association with spheroid formation in colorectal cancer

Running title: Expression of ALDOC in CRC

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Abbreviated words:

ALDOC, aldolase, fructose-bisphosphate C; CI, confidence interval; CRC, colorectal cancer; CSCs, cancer stem cells; F1,6BP Fructose 1,6-bisphosphate; G3P Glyceraldehyde 3-phosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA;

Abstract

Colorectal cancer is a leading cause of cancer-related death worldwide. The spheroid colony formation assay is a useful method to identify cancer stem cells. Using the DLD-1 and WiDr colorectal cancer cell lines, we performed microarray analyses of spheroid body-forming and parental cells and demonstrated that aldolase, fructose-bisphosphate C (ALDOC) was overexpressed in the spheroid body-forming cells of both lines. Cells transfected with small interfering RNA against ALDOC demonstrated lower proliferation, migration and invasion compared with negative control cells. Both the number and size of spheres produced by the colorectal cancer cells were significantly reduced by ALDOC knockdown. Additionally, inhibition of ALDOC reduced lactate production. Immunohistochemistry was used to analyze ALDOC protein expression in tissues from 135 colorectal cancer patients and revealed that 66 (49%) cases were positive for ALDOC. The ALDOC-positive cases were associated with higher T and M grades and, as determined by Kaplan-Meier analysis, a poorer prognosis. Univariate and multivariate analyses indicated that ALDOC expression was an independent prognostic factor for colorectal cancer patients. Furthermore, ALDOC expression was associated with CD44 expression. These results suggest that ALDOC contributes to colorectal cancer progression and plays an important role in cancer stem cells derived from colorectal cancer.

Keywords: colorectal cancer, ALDOC, spheroid, cancer stem cell

Introduction

Colorectal cancer (CRC) is one of the most common human cancers. Advances in diagnosis and treatments have improved the survival rate of patients with early-stage CRC. However, despite these advances, the survival rate of patients with recurrent or metastatic CRC remains poor. There is an urgent need to identify new diagnostic and therapeutic target molecules to develop novel CRC treatment strategies.

Cancer stem cells (CSCs) have been proposed as drivers of tumor initiation and self-renewal.[1] CSCs are closely associated with chemotherapy resistance, cancer recurrence, and metastasis.[1] Therefore, characterizing CSCs is important to establish more effective cancer treatments. One useful method for characterizing CSCs is the spheroid colony formation assay.[2] To form spheroid colonies, cells are cultured in serum-free media in culture dishes that are specifically coated to reduce attachment. The spheroid colonies display characteristics of the CSC phenotype.[2]

In the present study, we analyzed the gene expression profiles of spheroid colonies from two CRC cell lines using DNA microarrays. Microarray analysis revealed that expression of the aldolase, fructose-bisphosphate C (*ALDOC*) gene was upregulated in CRC spheroid colonies. ALDOC is a member of the aldolase family and is the fourth enzyme in the glycolytic pathway. ALDOC converts fructose 1,6-bisphosphate (F1,6BP) to glyceraldehyde 3-phosphate (G3P)

and dihydroxyacetone phosphate (DHAP).[3] Aldolase has been reported to be a sensor for glucose activity that regulates adenosine 5′ monophosphate-activated protein kinase (AMPK).[4] ALDOC overexpression has been described in multiple cancers, including breast,[5] gallbladder,[6] and gastric[7] cancers. ALDOC overexpression is more prevalent in aggressive than in less aggressive breast cancer subtypes.[5] In gallbladder cancer, ALDOC knockdown significantly downregulated glucose uptake, glycolysis, and cell proliferation.[6] Furthermore, knockdown of ALDOC reduced the migration of glioblastoma cells.[8]

The detailed function and expression profiles of ALDOC in CRC remain unknown. Therefore, we analyzed the expression and distribution of ALDOC in human CRC patient samples by immunohistochemistry and examined the relationship between ALDOC staining and clinicopathological characteristics. Furthermore, we analyzed the effect of inhibiting ALDOC expression in CRC cell lines.

Materials and methods

Tissue samples and cell lines

Surgically resected tumors (135) were collected and preserved from CRC patients who underwent curative resection between 2006 and 2010 at Hiroshima University Hospital (Hiroshima, Japan). Patients were only enrolled in this study if they were not preoperatively treated with radiotherapy or chemotherapy. Postoperative follow-up appointments were

scheduled every 3 months during the first 2 years, every 6 months during the following 3 years, and yearly thereafter. Because written informed consent was not obtained, for strict privacy protection, all of the identifying information associated with the samples was removed before the analysis. This study was approved by the Ethical Committee for Human Genome Research of Hiroshima University, Hiroshima, Japan (no. IRINHI66).

The human colon cancer-derived cell lines DLD-1, LoVo, WiDr, RKO and SW48 were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All cell lines were maintained at 37 °C in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd.; Tokyo, Japan) containing 10% fetal bovine serum (Corning; Corning, NY, USA) in a humidified atmosphere with 5% CO_2.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using ISOGEN (Nippon Gene, Tokyo, Japan) and 1 μg of total RNA was converted to cDNA with PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). PCR was conducted using the SYBR-Green PCR Master Mix (Thermo Fisher, Waltham, MA, USA). Real-time detection of the emission intensity of SYBR-Green bound to double-stranded DNA was performed with CFX Connect real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). ACTB-specific PCR products were amplified from the same RNA samples and served as an internal control. Primer sequences for ALDOC were

forward, 5′-GGATGAGTCTGTAGGCAGCAT-3′ and reverse, 5′-CACGACGATGCCCTTATCCT-3′. Primer sequences for ACTB were forward, 5′-CTGTCTGGCGGCACCACCAT-3′ and reverse, 5′-GCAACTAAGTCATAGTCCGC-3′

RNA interference (RNAi)

Small interfering RNA (siRNA) that targeted ALDOC and negative control oligonucleotides were purchased from Invitrogen (Carlsbad, CA, USA). We used 3 independent ALDOC-specific siRNA oligonucleotide sequences. Transfections of the CRC cell lines were performed using Lipofectamine RNAiMAX (Invitrogen) as previously described.[9] Briefly, 60 pmol of siRNA and 10 μL of Lipofectamine RNAiMAX were mixed in 1 mL of RPMI-1640 medium (final siRNA concentration of 10 nmol/L). After 20 min of incubation, the mixture was added to the cells, and the cells were plated in culture dishes. Forty-eight hours after transfection, the CRC cells were analyzed as described below.

Western blot analysis

Cells were lysed as previously described.[10] The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Following electrophoresis, proteins were transferred to nitrocellulose membranes and incubated with primary antibodies against ALDOC. Peroxidase-conjugated

anti-mouse or anti-rabbit IgG were used in the secondary reactions. Immunocomplexes were visualized with the ECL Western Blot Detection system (Amersham Biosciences Corp., Piscataway, NJ, USA). Anti-β-actin (Sigma-Aldrich) was used as the loading control.

Cell growth assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to examine cell growth. The CRC cells were seeded at a density of 3000 cells/well in 96-well plates. Cell growth was monitored after 1, 2, and 4 days. Three separate MTT experiments were performed and the mean ± standard deviation (SD) was calculated.

Spheroid colony formation assay

For the generation of spheroids, 1000 cells were seeded in six-well ultra-low attachment plates (Corning). Cells were grown in mTeSR medium (STEMCELL Technologies Inc.; Vancouver, BC, Canada). The plates were incubated at 37 °C in an incubator with a 5% CO_2 atmosphere for 15 days. Spheroid numbers and sizes were determined using a microscope.

Cell migration assay

To monitor cell migration, wound healing assays were performed with the ibidi 2 Well Culture-Insert (ibidi GmbH, Gräfelfing, Germany). The CRC cells were suspended at a concentration

of 1×10^6 cells/mL in RPMI medium, and 70 μL of cells were added to each well. After 24 hours, the inserts were gently removed, and the cells were cultured with serum-free RPMI medium. Images were obtained at the appropriate times using phase contrast microscopy.

Invasion assay

A modified Boyden chamber assay was performed to examine cell invasiveness. 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; Corning, NY, USA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber. After incubation at 37°C for 48 h, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained with CyQuant GR dye (Chemicon, Temecula, CA, USA) to assess the number of cells.

Lactate assay

Cells were seeded at a density of 1×10^5 cells/well in 6 well plates. After 24 h, the medium was collected from each well. Lactate concentrations were measured using the Lactate Assay Kit (Cell Biolabs, Inc.; San Diego, CA, USA) in accordance with the manufacturer's protocol.

Immunohistochemistry

One or two representative tumor blocks that included the tumor center, invasive front, and tumor-associated non-neoplastic mucosa were examined for each patient by immunohistochemistry. In cases of large late-stage tumors, two different sections were examined to include representative areas of the tumor center as well as the lateral and deep tumor invasive front. Immunohistochemical analysis was performed using the Dako EnVision+ Peroxidase Detection System (DakoCytomation; Carpinteria, CA, USA). Antigen retrieval was performed by microwave heating the sections in citrate buffer (pH 6.0) for 30 min. Peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, and non-specific antibody binding sites were blocked by incubating the sections with normal goat serum (DakoCytomation) for 20 min. The sections were incubated with rabbit polyclonal anti-ALDOC antibody (1:400, product number AV48273; Sigma-Aldrich; St Louis, MO, USA) or mouse monoclonal anti-CD44 antibody (1:100, clone DF1485; Novocastra; Newcastle upon Tyne, UK) for 1 h at room temperature, followed by incubation with EnVision+ peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies, respectively, for 1 h. For the color reaction, sections were incubated with the DAB substrate-chromogen solution (DakoCytomation) for 5 min. Sections were counterstained with 0.1% hematoxylin. Negative controls were created by omitting the primary antibody. The CRC cases with staining of >20% of tumor cells were regarded as positive because the median positivity rate in the CRC cells was 20%. Using this definition, the immunoreactivity in each specimen was independently reviewed by two surgical pathologists

(NO and KS) who had no knowledge of clinicopathological parameters or patient outcomes. After independent reviews, interobserver differences were resolved by consensus review using a double-headed microscope.

Statistical analysis

Associations between clinicopathological parameters and ALDOC expression were analyzed by Fisher's exact test. Kaplan-Meier survival curves were constructed for ALDOC-positive and ALDOC-negative patients. Univariate and multivariate Cox regression analyses were used to evaluate the associations between clinical covariates and survival. Differences between the two groups were tested using the Student t-test. The results are expressed as the mean ± SD of triplicate measurements. JMP Pro, version 15.0.0, (SAS Institute Inc.; Cary, NC, USA) was used for these analyses. A p-value of less than 0.05 was considered statistically significant.

Results

Gene expression profiles of the spheroid body-forming and parental cells

We performed microarray analyses of spheroid body-forming and attached parental DLD-1 and WiDr cells using Clariom D microarrays to characterize the spheroid colonies. In DLD-1 cells, we found 1058 genes that were significantly upregulated in the spheroid body-forming cells compared with the attached parental cells. We also found 958 genes that were significantly

downregulated in the spheroid body-forming cells in comparison to the parental cells. In WiDr cells, microarray analysis revealed that 1038 genes were significantly upregulated and 986 genes were significantly downregulated in the spheroid body-forming cells compared with the attached parental cells. To identify ideal biomarkers for CRC stem cells, we focused on genes that were highly expressed in the spheroid body-forming cells from both DLD-1 and WiDr cells and identified 336 genes. Among these 336 genes, ALDOC expression was higher in CRC than in normal colorectal tissue in TCGA database (Supporting Fig. S1). To our knowledge, detailed function and expression profiles of the ALDOC gene in human CRC remain to be analyzed. Thus, we focused on ALDOC. First, we confirmed that ALDOC mRNA expression was up-regulated in spheroids body-forming cells compared with attached parental cells by qRT-PCR. (Fig. 1a).

Effect of inhibition of ALDOC on cell growth

The biological function of ALDOC was investigated using CRC cell lines. Western blot analysis revealed various levels of ALDOC expression in CRC lines (Fig. 1b). LoVo and RKO cells showed high ALDOC expression and were selected for further experiments. The expression of ALDOC was substantially suppressed by treatment with ALDOC-specific siRNA2 and siRNA3, as observed by Western blot (Fig. 1c). We performed MTT assays 1, 2, and 4 day after siRNA transfection to investigate the antiproliferative effects of ALDOC inhibition. ALDOC siRNA2-

and siRNA3-transfected LoVo and RKO cells showed significantly reduced cell growth relative to the negative control siRNA-transfected cells (Fig. 1d). These results indicate that ALDOC is involved in CRC cell growth.

Next, we analyzed the association between ALDOC expression and spheroid formation in CRC cells. Both the number and size of spheroids were significantly reduced in ALDOC siRNA-transfected cells compared with the control cells (Fig. 1e). These results suggest that ALDOC plays an important role in the formation of spheroids.

Effect of inhibition of ALDOC on cell migration and invasion.

We investigated the possible role of ALDOC in migration of CRC cells. The migration activities of LoVo and RKO cells transfected with ALDOC siRNAs were lower than the cells transfected with negative control siRNA (Fig. 2a, b). Next, we performed invasion assay to determine the possible role of ALDOC in the invasiveness of CRC cells. The numbers of invaded LoVo and RKO cells transfected with ALDOC siRNAs were significantly fewer than the cells transfected with negative control siRNA (Fig. 2c, d). These results indicate that ALDOC may play an important role in tumor migration and invasion.

Effect of inhibition of ALDOC on the glycolytic pathway

ALDOC is an enzyme that regulates the glycolytic system.[3] In addition, it has been reported

that ALDOA, a family member protein, enhances glycolysis in colon cancer.[11] Therefore, we investigated the effect of ALDOC suppression on glycolysis in colon cancer cells. In LoVo and RKO cells, the knockdown of ALDOC resulted in a decrease in lactate production compared with the controls (Fig. 2e, f). These results support a positive correlation between ALDOC expression and glycolysis.

Expression of ALDOC in CRC tissue samples

To confirm the clinical significance of ALDOC, we performed immunohistochemical analysis of 135 surgically resected CRC tissue samples. In non-neoplastic colonic mucosa, weak or no staining of ALDOC was observed in the foveolar epithelium and stromal cells. In contrast, CRC tissue showed stronger, more extensive staining. Staining of ALDOC was observed mainly in the cytoplasm (Fig. 3a). The CRC tissues showed heterogeneity of ALDOC staining, and the percentage of ALDOC-stained CRC cells ranged from 0% to 70%. Upregulation of ALDOC at the invasive front was not observed. When > 20% of tumor cells were stained, the immunostaining was considered positive for ALDOC. In total, 66 (49%) of the 135 CRC cases were ALDOC-positive.

We examined the relationship between ALDOC expression and clinicopathological characteristics. ALDOC-positive cases were associated with T and M stages (Table 1) and showed significantly poorer overall survival (Fig. 3b). Furthermore, univariate and multivariate

Cox proportional hazards analyses demonstrated that ALDOC expression was an independent prognostic factor for overall survival in CRC patients (Table 2). These results indicate that ALDOC expression is associated with CRC progression.

Association between ALDOC and CSC marker expression

We investigated whether ALDOC expression was associated with CRC stemness. Since CD44 is a representative CSC marker in CRC patients, we performed immunohistochemical staining for CD44 in the 135 CRC tissue samples. CD44 staining was observed mainly in cellular membranes within the CRC tissue (Fig. 3c). In total, 70 (52%) of the 135 CRC cases were CD44-positive. Comparisons of staining patterns showed that ALDOC and CD44 tended to be expressed in the same tumor cells (Fig. 3c). Furthermore, ALDOC-positive CRC cases were frequently found among CD44-positive CRC cases (Table 3). These results indicate that ALDOC expression may be involved in CRC stemness.

Discussion

CSCs are defined as a unique subpopulation of cells that possess self-renewal and differentiation potential. CSCs are generally responsible for tumor initiation, invasion, metastasis, and chemoresistance.[12] Thus, CSC characterization can improve our understanding of carcinogenesis and help advance the treatment of CRC.

To characterize CSCs, one useful method is the spheroid colony formation assay.[2] Spheroid colonies have characteristics that resemble the CSC phenotype.[2] It has been reported that CD44 is upregulated during spheroid formation,[13] and this protein has been identified as one of the cell surface markers associated with CSCs.[14] These results suggest that genes that are upregulated during spheroid formation may be markers for CSCs.

In this study, we identified ALDOC as a gene that is overexpressed in spheroid-forming cells. ALDOC converts F1,6BP to G3P and DHAP and can also convert fructose 1-phosphate to DHAP and glyceraldehyde.[3] However, the association between ALDOC and CSC in CRC remains unknown. We demonstrated that both the number and size of spheres formed by CRC cell lines were significantly reduced in ALDOC siRNA-transfected cells in comparison with negative control cells. These results suggest that ALDOC is required for sphere formation in CRC cells. However, the underlying mechanisms between the stemness of CRC and this gene remain unclear. Further studies are needed to clarify the biological function of ALDOC in CRC stem cells.

Immunostaining results showed that ALDOC was overexpressed in 49% of colorectal cancer cases, and there was a significant correlation between ALDOC expression and the T grade. Biological function analysis showed that cell proliferation was decreased in ALDOC-knockdown cells compared with negative control cells, suggesting that ALDOC is involved in carcinogenesis of CRC. Additionally, we showed a correlation between ALDOC expression

and a high M grade in CRC tissues. These results were collaborated by ALDOC knockdown CRC cell lines. Therefore, ALDOC may also contribute to the metastasis of CRC.

As noted above, ALDOC appears to be required for the formation of CRC cell spheroids. Furthermore, immunohistochemical staining showed that the expression of ALDOC was predominantly correlated with the expression of CD44. These results suggest that ALDOC may be instrumental in the production of CSCs in CRC. Since CSCs are characterized as a minority population (< 5% of CRC cells),[15] ALDOC is not a specific marker for colorectal CSC. Further studies will be required to clarify the biological functions of ALDOC in CRC stem cells.

Enhanced glycolysis is one of the hallmarks of cancer.[16] In cancer cells, elevated glucose uptake and increased lactate production are observed. Increased glycolysis is associated with the activation of oncogenes and mutations in tumor suppressors. These changes in cancer cells mainly cause increased cell proliferation, evasion of growth inhibition, and attenuation of apoptosis.[17] In this study, we showed that suppression of ALDOC in CRC cell lines decreased lactate production. These findings suggest that ALDOC may contribute to the malignant transformation of colon cancer by enhancing glycolysis. Recently, it was reported that ALDOC is a glucose sensor.[4,18] ALDOC that is unoccupied by F1,6BP may activate AMPK and contribute to cancer survival. Mucin 16 was reported to stabilize ALDOC in gallbladder carcinoma and increase the accumulation of unoccupied ALDOC, which activated the AMPK pathway and promoted glycolysis and proliferation.[6] The mechanism by which ALDOC

regulates CRC malignancy through the glycolytic system requires further investigation.

ALDOC is not only involved in the glycolytic system, but also functions to activate the WNT signaling pathway.[19] ALDOC binds to glycogen synthase kinase-3β and inhibits the formation of the β-catenin degradation complex.[19] Consequently, the β-catenin protein is stabilized and transferred to the nucleus to activate downstream factors involved in tumorigenesis.[19] In this study, immunostaining showed no correlation between ALDOC expression and nuclear translocation of β-catenin (data not shown). In a previous study, alterations in the WNT signaling pathway was reported in 93% of CRC cases.[20] Therefore, activation of WNT signaling may occur via an ALDOC-independent pathway. Further investigation of the ALDOC and WNT signaling pathways will be needed to determine if there is an association between ALDOC expression and nuclear translocation of β-catenin in CRC cases without adenomatous polyposis coli or β-catenin mutations. Because only knockdown of ALDOC was performed in the present study, we should perform similar series of studies by using CRC cells with ALDOC overexpression in the near future to analyze ALDOC function in CRC cells.

In summary, we found that ALDOC is overexpressed in CRC and represents an independent prognostic factor for patients with CRC. We also revealed that knockdown of ALDOC by RNAi inhibited cell growth, migration, and spheroid formation. Thus, ALDOC likely plays an important role in CSC and may have potential as a predictive diagnostic and therapeutic target for CRC.

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Disclosure statement

None declared.

Author Contributions

RM (first author), NO (corresponding author) conceptualized and designed the study. RM and YN performed data collection and analyses. SA, YF, DT, and KS assisted with the molecular technologies that were used. RM drafted the manuscript. AI contributed to the preparation of the revised manuscript. NO provided input regarding manuscript preparation. All authors have read and approved the final manuscript.

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Table 1 Relationship between ALDOC expression and clinicopathologic characteristics

	ALDOC expression		
	Positive	Negative	p-value
Age			
≤65 (n=63)	36 (57%)	27	0.0722
>65 (n=72)	30	42	
Sex			
Male (n=83)	39 (47%)	44	0.5767
Female (n=52)	27	25	
Tumor Location			
Right colon (n=39)	21 (54%)	18	0.7068
Left colon (n=96)	45	51	
Differentiation			
Differentiated (n=122)	59 (48%)	63	0.4626
Un-differentiated (n=13)	7	6	
T grade			
T1/T2 (n=40)	13 (33%)	27	0.0127
T3/T4 (n=95)	53	42	
N grade			
N0 (n=62)	26 (42%)	36	0.1357
N1/N2/N3 (n=73)	40	33	
M grade			
M0 (n=107)	46 (43%)	61	0.0067
M1 (n=28)	20	8	
Lymphatic invasion			
ly0 (n=59)	25 (42%)	34	0.1814
ly1/2/3 (n=76)	41	35	
Vascular invasion			
v0 (n=66)	31 (47%)	35	0.6626
v1/2/3 (n=69)	35	34	
Stage			
Stage I/II (n=58)	24 (41%)	34	0.1291
Stage III/IV (n=77)	42	35	

p-values were calculated with Chi-square test.

Table 2 Univariate and multivariate Cox regression analysis of survival

	Univariate analysis			Multivariate analysis		
	HR	95 % CI	p-value	HR	95 % CI	p-value
Age						
≤65	1 (Ref.)					
>65	1.128	(0.586-2.170)	0.7182			
Sex						
Male	1 (Ref.)					
Female	0.595	(0.293-1.210)	0.1403			
Tumor Location						
Right colon	1 (Ref.)					
Left colon	0.641	(0.325-1.267)	0.212			
Differentiation						
Differentiated	1 (Ref.)					
Un-differentiated	1.084	(0.332-3.541)	0.8948			
T grade						
T1/T2	1 (Ref.)			1(Ref.)		
T3/T4	7.865	(1.888-32.765)	0.0001	1.61	(0.336-7.728)	0.5515
N grade						
N0	1 (Ref.)			1 (Ref.)		
N1/N2/N3	5.227	(2.172-12.577)	<0.001	0.482	(0.132-1.004)	0.2695
M grade						
M0	1 (Ref.)			1 (Ref.)		
M1	14.001	(6.825-28.720)	< 0.001	6.146	(2.714-13.916)	< 0.001
Lymphatic invasion						
ly0	1 (Ref.)			1 (Ref.)		
ly1/2/3	3.05	(1.432-6.493)	0.0019	1.918	(0.843-4.363)	0.1206
Vascular invasion						
v0	1 (Ref.)					
v1/2/3	1.867	(0.805-3.736)	0.0697			
Stage						
Stage I/II	1 (Ref.)			1 (Ref.)		
Stage III/IV	10.984	(3.363-35.881)	< 0.001	6.98	(1.129-43.160)	0.0436
ALDOC expression						
Negative	1 (Ref.)			1 (Ref.)		
Positive	3.104	(1.521-6.337)	0.0011	2.126	(1.010-4.476)	0.0400

HR, hazard ratio; CI, confidence interval.

Table 3 Correlation between ALDOC expression and CD44

	ALDOC expression		
	Positive	Negative	p-value
CD44			0.0091
Positive	41(59%)	29	
Negative	25(38%)	40	

p-values were calculated with Chi-square test.

Figure legends

Figure 1. Effects of ALDOC inhibition on cell growth and spheroid formation in colorectal cancer (CRC) cell lines. (a) qRT-PCR analysis of ALDOC in DLD-1 and WiDr. Fold-change indicates the ratio of ALDOC mRNA expression in spheroid body-forming cells to that in attached parental cells. (b) Western blot analysis of ALDOC in five CRC cell lines. (c) Western blot analysis of ALDOC in RKO and LoVo cells transfected with the ALDOC-specific siRNA and negative control siRNA. (d) Cell growth was assessed by MTT assays at days 1, 2, and 4 after transfection and seeding in 96-well plates. Data are presented as means ± standard deviations of 3 independent experiments. * $p < 0.05$. (e) The numbers and size of spheroids formed by RKO and LoVo cells transfected with ALDOC-specific or negative control siRNAs. Data are presented as means ± standard deviations of 3 independent experiments. * $p < 0.05$.

Figure 2. Effects of ALDOC inhibition on migration of and lactate production in CRC cells. (a) Wound healing assays for RKO cells transfected with the ALDOC siRNA or negative control siRNA. The migration of cells was captured at 0 h and 24 h after scratching. (b) Wound healing assays for LoVo cells transfected with the ALDOC siRNA or negative control siRNA. The migration of cells was captured at 0 h and 48 h after scratching. (c) Images of invasion assay in RKO cells transfected with the ALDOC siRNA or negative control siRNA at 48 h and

quantification of the average number of invaded cells. (d) Images of invasion assay in LoVo cells transfected with the ALDOC siRNA or negative control siRNA at 48 h and quantification of the average number of invaded cells. (e) RKO cells and (f) LoVo cells transfected with the ALDOC siRNA and negative control siRNA were cultured for 1 day, and the lactate concentration in the culture medium was analyzed. Data are presented as means ± standard deviations of 3 independent experiments. * $p < 0.05$.

Figure 3. Immunohistochemical analysis of ALDOC in colorectal cancer (CRC) tissue samples. (a) Immunohistochemical analysis of ALDOC in CRC. Left panel, original magnification, ×40. Right panel, a high-magnification image of the field designated by the box in the left panel; original magnification, ×400. (b) Kaplan-Meier plots of the survival of CRC patients. (c) Immunohistochemical analysis of ALDOC and CD44. Original magnification, ×400.

Figure 1

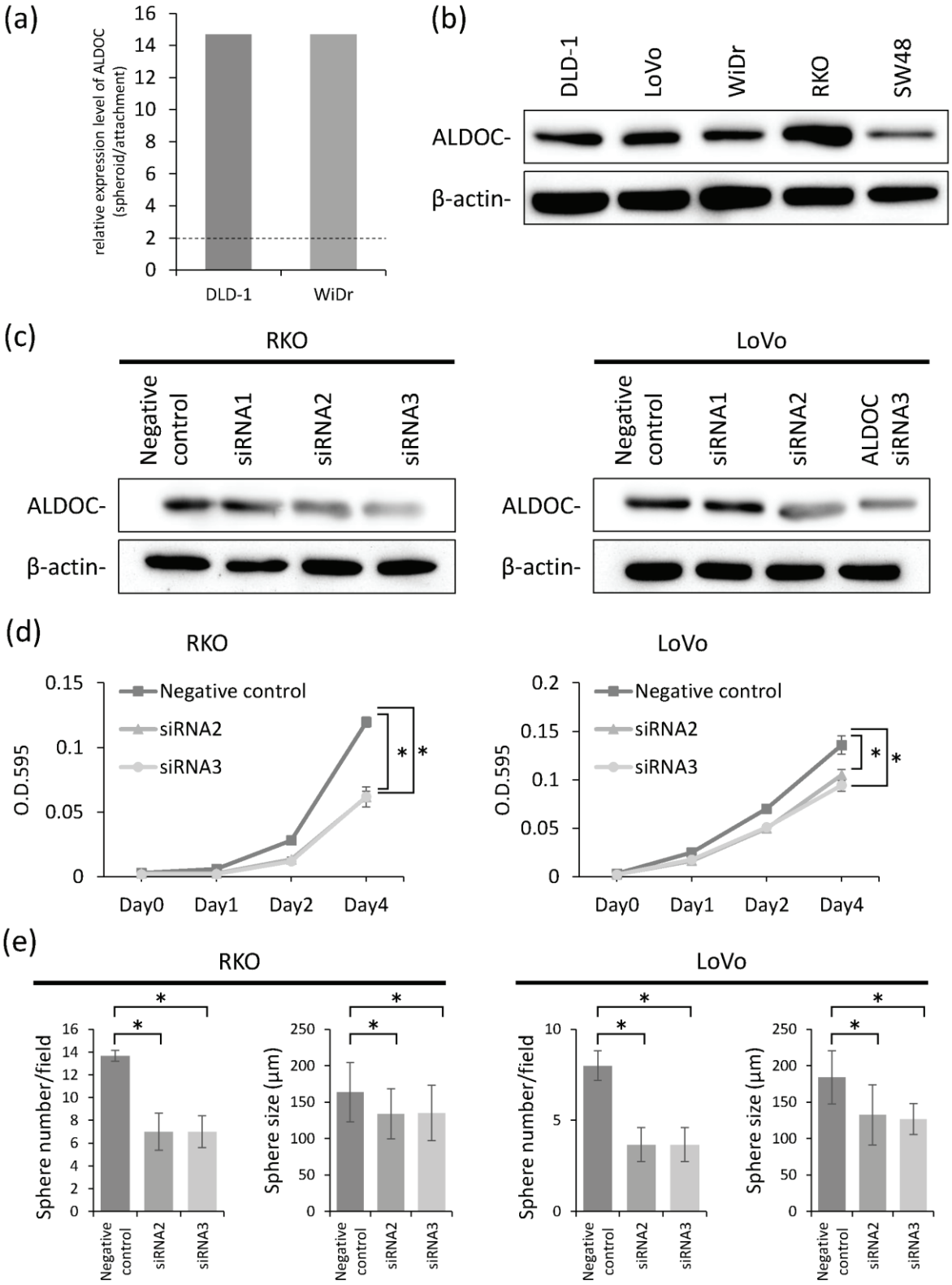

(a)
relative expression level of ALDOC (spheroid/attachment)
16
14
12
10
8
6
4
2
0
DLD-1
WiDr
(b)
DLD-1
LoVo
WiDr
RKO
SW48
ALDOC-
β-actin-
(c)
RKO
Negative control
siRNA1
siRNA2
siRNA3
ALDOC-
β-actin-
LoVo
Negative control
siRNA1
siRNA2
ALDOC siRNA3
ALDOC-
β-actin-
(d)
RKO
0.15
0.1
0.05
0
O.D.595
Negative control
siRNA2
siRNA3
* *
Day0
Day1
Day2
Day4
LoVo
0.2
0.15
0.1
0.05
0
O.D.595
Negative control
siRNA2
siRNA3
* *
Day0
Day1
Day2
Day4
(e)
RKO
Sphere number/field
16
14
12
10
8
6
4
2
0
* *
Negative control
siRNA2
siRNA3
Sphere size (μm)
250
200
150
100
50
0
* *
Negative control
siRNA2
siRNA3
LoVo
Sphere number/field
10
5
0
* *
Negative control
siRNA2
siRNA3
Sphere size (μm)
250
200
150
100
50
0
* *
Negative control
siRNA2
siRNA3

Figure 2

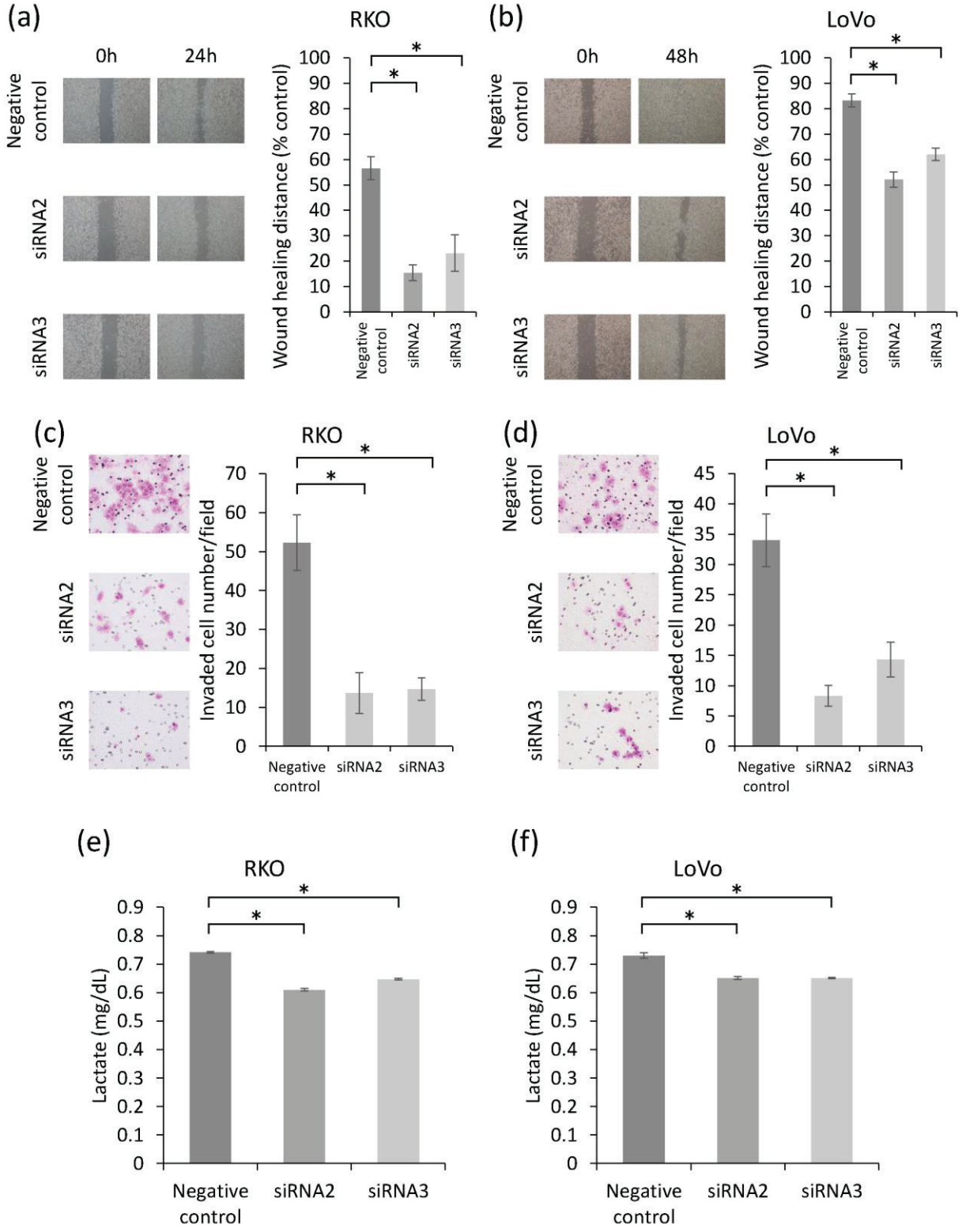
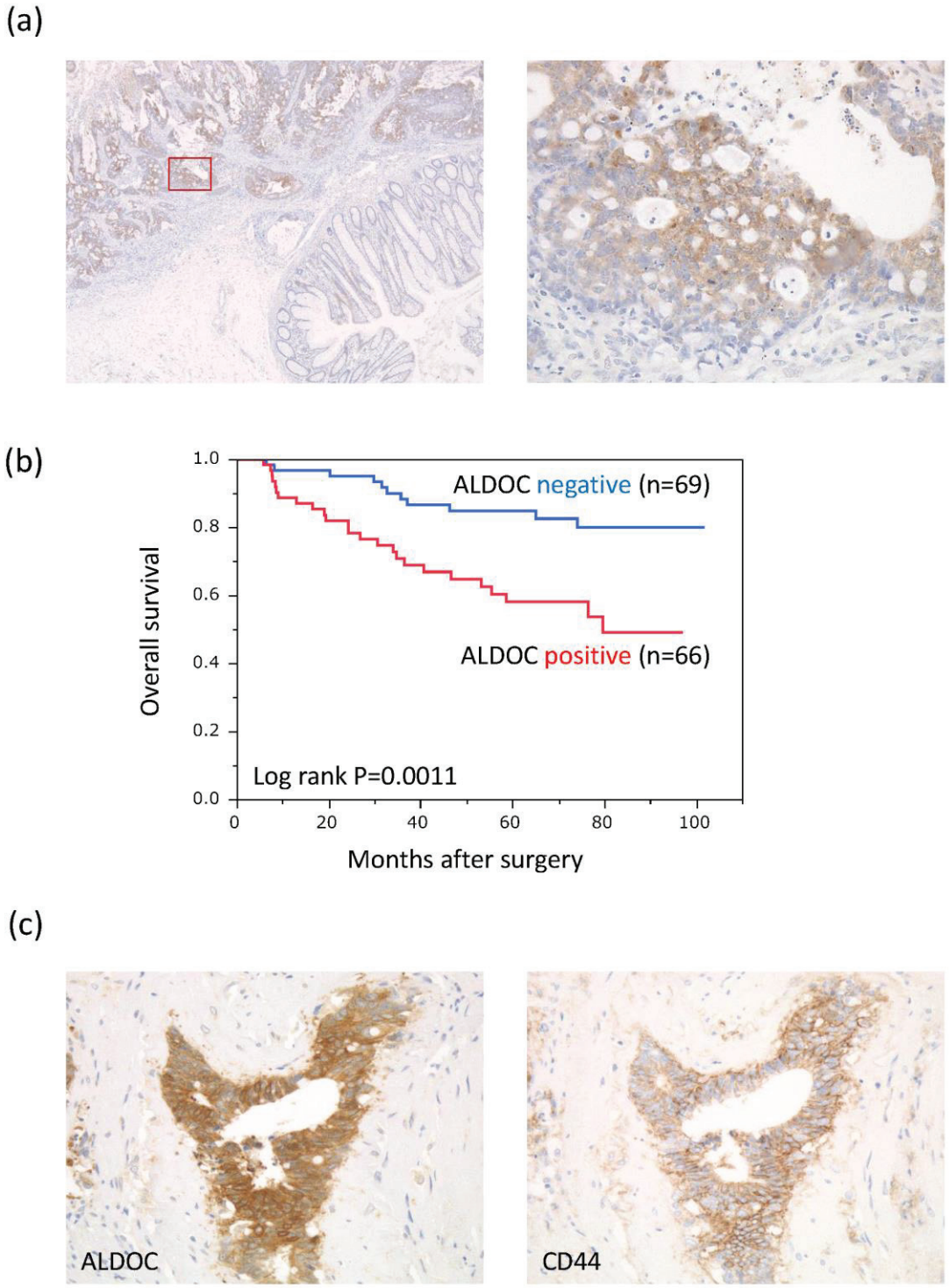

(a)
0h
24h
Negative control
siRNA2
siRNA3
RKO
Wound healing distance (% control)
Negative control
siRNA2
siRNA3
(b)
0h
48h
LoVo
(c)
Invaded cell number/field
(d)
(e)
Lactate (mg/dL)
(f)

Figure 3


(a)
(b)
1.0
0.8
0.6
0.4
0.2
0.0
Overall survival
ALDOC negative (n=69)
ALDOC positive (n=66)
Log rank P=0.0011
0
20
40
60
80
100
Months after surgery
(c)
ALDOC
CD44