

Original Article

Role and mechanism of ALDH1A1 expression in the development of gastric cancer

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Abstract: ALDH1A1 is one member of the ALDHs gene superfamily locating on human chromosome 9, which oxidizes various aldehydes to the corresponding carboxylic acids. The study aimed to further validate ALDH1A1 expression in gastric cancer tissues and adjacent tissues, and to screen the cell line with highest expression among 6 strains of gastric cancer cell lines and transfected it into gastric cancer cells by the construction of siRNA-ALDH1A1 expression vector. We explored the effect of ALDH1A1 expression on the proliferation, migration and invasion of gastric cancer cells by MTT test, flow cytometry, scratch test and invasive experiments, and detected Wnt/ β -catenin signaling pathway activation by western blot technology. The expression level of the ALDH1A1 in gastric cancer tissue (75.0%, 60/80) was higher than para-carcinoma tissue (38.8%, 31/80) ($P < 0.05$). The cell migration distance in the ALDH1A1 transfection group was longer than that in the control group (42.10 vs. 22.38, $P < 0.01$). The number of gastric cancer cells through the inferior vena in ALDH1A1 transfection group and was more than that in the control group (99.29 vs. 59.37, $P < 0.05$). The siRNA-ALDH1A1 significantly inhibited the expression of Wnt1, Wnt2 and β -catenin in the gastric cells, the expression of CyclinD1 and PCNA and the expression of MMP-2 & MMP-9 compared to the control group (all $P < 0.01$). ALDH1A1 also up-regulated the expression of E-cadherin and down-regulate the expression of N-cadherin in the gastric cells ($P < 0.01$). In conclusion, interfering ALDH1A1 expression could significantly inhibit the proliferation, migration, invasion and metastasis of MKN-45, and block the cell cycle in phase G1, which were achieved by down-regulating CyclinD1, PCNA, MMP-2, MMP-9 and N-cadherin expression, up-regulating the expression of E-cadherin, and reduce the expressions of Wnt1, Wnt2 and β -catenin.

Keywords: ALDH1A1, gastric cancer, proliferation, migration, invasion

Introduction

Gastric cancer has high morbidity and mortality throughout the world, which is the same in China with the 5-year survival rate less than 30% and seriously threatening people's life and health [1]. Therefore, it is of great significance to explore the pathogenesis of gastric cancer, and to seek new tumor markers and the gene therapy targets of gastric cancer, thus improving the diagnostic level and prognosis of gastric cancer. The occurrence and development of gastric cancer development is a typical multi-factor and multi-step process, which involves the disorder of the key signaling pathways in the regulating cell, including cell proliferation, apoptosis, invasion and metastasis. Wnt signaling pathway is highly conservative involving in the evolution of multicellular eukaryotes, regulating various multicellular eukaryotes life

activities. Disorders of Wnt signaling pathway are closely related to the occurrence and development of tumors, such as cervical cancer, breast cancer, gastric cancer, liver cancer, prostate cancer, bladder cancer and so on [2-5]. β -serial protein (β -catenin) mediated pathway is a typical one among the Wnt signaling pathways. In normal physiological circumstances, β -catenin as cytoskeleton protein forms a complex with the E-calcium adhesion factor (E-cadherin) to maintain homotopic cell adhesion and prevent the movement of cells. When stimulated by upstream signal, β -catenin was accumulated in the cytoplasm by inhibiting the phosphorylation mediated by glycogen synthase kinase 3 β (GSK3 β) and transferred to the nucleus, regulating downstream gene expression, such as cell cycle protein D1 (CyclinD1), matrix metalloproteinases (MMPs), E-cadherin and N-cadherin protein, thereby leading to the

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occurrence of tumor. Wnt/ β -catenin signaling pathway was activated in most patients with gastric cancer [6, 7].

ALDH1A1 is one member of the ALDHs (aldehyde dehydrogenases, acetaldehyde dehydrogenase) gene superfamily locating on human chromosome 9, which could oxidize various aldehydes to the corresponding carboxylic acids. ALDH1A1 protein is the autotetraploid somatic cell plasma protein, expressing in the brain of the cornea, lens, retina, hepatic and gastrointestinal epithelial tissues [8]. Li, et al. determined 216 gastric cancer tissue samples by immunohistochemical technology and found high expression of ALDH1A1 [9]. Wu et al. determined the expression of ALDH1A1 in 1072 cases of gastric cancer tissues and adjacent tissues, revealing that ALDH1A1 expression in gastric cancer tissues was significantly higher than that of adjacent tissues [10]. Highly active ALDH1A1 in breast cancer, prostate cancer and bladder cancer cells had increased abilities of clone, tumor formation and migration [2-5]. siRNA interfered ALDH1A1 expression of drug-resistant ovarian cancer cells, which obviously inhibited tumor growth and migration in nude mice compared to single chemotherapy [11]. Wnt/ β -catenin signaling pathway was inhibited, thereby reducing ALDH1A1 the number of positive prostate cancer cells, and improving the effect of chemotherapy [12], which indicated that ALDH1A1 expression was closely related to the occurrence and development of a variety of malignant tumor.

Therefore, our study aimed to further validate ALDH1A1 expression in gastric cancer tissues and adjacent tissues, and to screen the cell line with highest expression among 6 strains of gastric cancer cell lines and transfected it into gastric cancer cells by the construction of siRNA-ALDH1A1 expression vector. We explored the effect of ALDH1A1 expression on the proliferation, migration and invasion of gastric cancer cells by MTT test, flow cytometry, scratch test and invasive experiments, and detected Wnt/ β -catenin signaling pathway activation by western blot technology.

Materials and methods

Cell lines

The MKN-45, MKN-28, AGS, BGC-823, SGC-7901, HGC-27 human gastric cancer cell lines

and an immortalized gastric mucosa epithelial cell line were purchased from cell bank, Chinese academy of sciences.

Reagents and instruments

MTT (Sigma Corp, USA), Rabbit CyclinD1, PCNA, MMP-2, MMP-9, E-cadherin, N-cadherin, Wnt2, GADPH Monoclonal Antibodies (Epitomics Corp), Rabbit Wnt1, β -catenin Monoclonal Antibodies (Epitomics); Cell cycle kit (Abcam Corp); Transwell Chambers (Corning Corp); Crystal violet (Sigma Corp). CO₂ Incubators (Thermo Scientific Corp); inverted microscope (Nikon Corp); Flow Cytometry (BD Corp); Mini double vertical electrophoresis apparatus, Mini transfer electrophoresis apparatus, ChemiDocTM XRS gel imaging system (Bio-Rad Corp).

Immunohistochemical detection of ALDH1A1 expression

The carcinoma tissue and normal tissue adjacent to carcinoma were surgically obtained from stomach cancer patients from our hospital. The samples were dewaxed by xylol and washed by anhydrous, 95%, 80% ethanol dehydration, and then closed with horse serum. The samples were soaked with wood grain, hydrochloric acid, and alcohol sequentially. And then they were watered until to blue and dehydrated by alcohol and transparentized by xylol. ALDH1A1 positive was stained for light yellow, tan or brown, and located in cytoplasm.

Detection the expression of ALDH1A1

RNA was extracted from samples using the operation instruction of Trizol Reagent box (Invitrogen) and in the environment without RNAase. Snail gene were amplified (F: 5'-CTCGAGATGTCATCCTCAG-3'; R: 5'-GAATTCGTGAGTTCTTCTG-3'). The cDNA sample was synthesized by using one-step RT-PCR kit and PCR amplification. 5 ul amplification products gained for detection in the next step of 2% agarose gel. Detected electrophoresis bands and took photos by ultraviolet spectrophotometer. Expression of protein was determined Western Blot with Protein Assay Kit.

Vector construction and cell transfection

The sh-RNA-ALDH1A1 carrier was synthesized by Gemma biotech companies, Shanghai.

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siRNA sequence of ALDH1A1 was 5'-GTAG-CCTTCACAGGATCAA-3' and control sequence was -TTCTCGGAACGTGTACAGT-3'. When the confluence of cells reached 30~50%, Vehicles were transfection by LipofectamineTM2000. The siRNA (250 μ l) was diluted with moderate amount of serum-free Opti-MEM[®] I medium, and gently blending. Shook up LipofectamineTM2000 was took a moderate amount to diluted and mixed with Opti-MEM[®] I, incubated for 5 min at room temperature. The diluted LipofectamineTM2000 and diluted siRNA were mixed and incubated for 20~30 min at room temperature. These siRNA/LipofectamineTM2000 complexes were inoculated in 6-well plates and mixed, changed to DMEM medium containing 10% serum after 6 h; cultured in 37°C, CO₂ incubator for 24~72 h.

Cell proliferation rate detection by MTT method

Cells were inoculated in 96-well plates, 100 μ l in each well and four plates for each group. When the confluence of cells reached 50%, siRNA-ALDH1A1 and corresponding control NC were transfected. When transfected by 48 hours, the cells were added 20 μ l 5 mg/ml MTT and abandoned culture solution after continued to be cultured for 4 hours, added DMSO 150 μ l in each well, shocked to dissolve the crystals, and detected the OD values at 560 nm of ELIASA and calculated relative growth rate with 630 nm as a reference wavelength.

Cell cycle detection by flow cytometry method

Cells were inoculated in 96-well plates, 100 μ l in each well and four plates for each group. When the confluence of cells reached 50%, siRNA-ALDH1A1 and corresponding control NC were transfected. When transfected by 48 hours, the protein concentration was measured according to the Cell cycle detection kit instruction. The samples placed at 4°C for 48 h after fixing. Before detection, the cells were washed, blended, added 5 μ l Rnase (10 mg/ml), incubated in 5% CO₂ incubator for 1 h, in which PI (100 μ g/ml) 200 μ L was added later, stained under the conditions of protection from light for 30 min at room temperature. Cell cycle was detected and analyzed by flow cytometry method.

Scratch test

Cells were inoculated in 96-well plates, 100 μ l in each well and four plates for each group.

When the confluence of cells reached 50%, siRNA-ALDH1A1 and corresponding control NC were transfected, When transfected by 48 hours, lined on 6 orifice cells using 20 μ l liquid move spear, washed three times using PBS, then cultured with serum-free RPMI-1640, observed measured the scratch width under Nikon inverted microscope, and took photos.

Cell invasion experiment

The Matrigel glue was evenly spread on the micro-film of the Transwell chamber, and was made as a gel spare. Cells were inoculated in 96-well plates, 100 μ l in each well and four plates for each group. When the confluence of cells reached 50%, siRNA-ALDH1A1 and corresponding control NC were transfected. When transfected by 48 hours, the cells were digested and added into Transwell upper chamber, and the lower chamber continued to be cultured for 24 hours in DMEM medium 5% fetal bovine serum. Then the Transwell chamber was removed and washed, fixed with paraformaldehyde, stained with crystal violet, counting the number of five fields of view of passed membrane cells under an inverted optical microscope and calculating the average number of cells per field of view, which represented the migration ability of cells.

Western blot

When the confluence of cells siRNA-ALDH1A1 and corresponding control NC were transfected, when transfected by 48 hours, the cells were scraped and centrifuged. After added appropriate amount of RIPA lysis buffer, the cells were put into the vortex meter and shocked for 30 seconds. After 40 minutes, they were centrifuged (4°C, 10000 rpm) for 10 minutes and carefully absorbed the supernatant, by which could obtain the total protein. The protein concentration was measured according to the BCA kit. The proteins ran SDS gel electrophoresis, and then wet brick membrane. The membrane was immersed and incubated in primary antibody solution and overnight in 4°C. After rinsed, proteins were immersed and incubated in the secondary antibody solution at room temperature for 1 to 2 hours. The membrane was removed and rinsed, on which ECL solution was dropped, exposed in the gel imaging system. Each antibody striped gray value was detected with "Quantity one" software.

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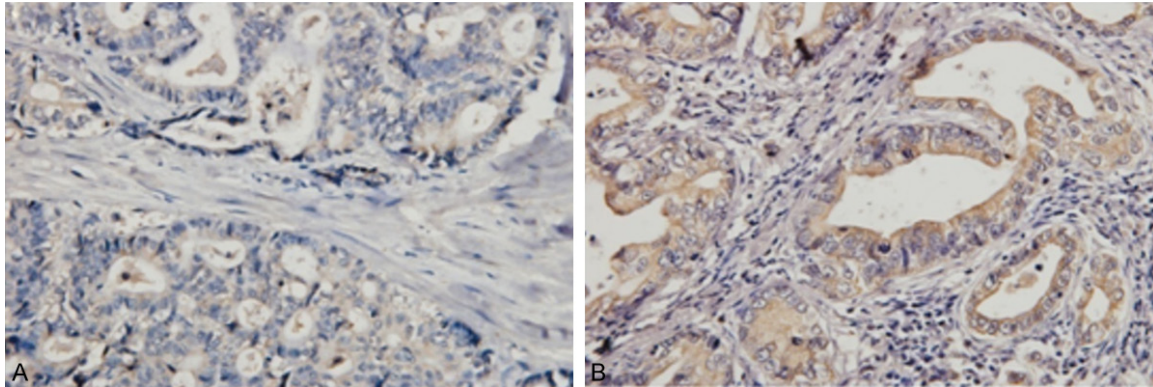


Figure 1. Expression level of the ALDH1A1 in gastric cancer tissue and para-carcinoma tissue of immunohistochemical detection. A: Para-carcinoma tissue, B: Gastric cancer tissue.

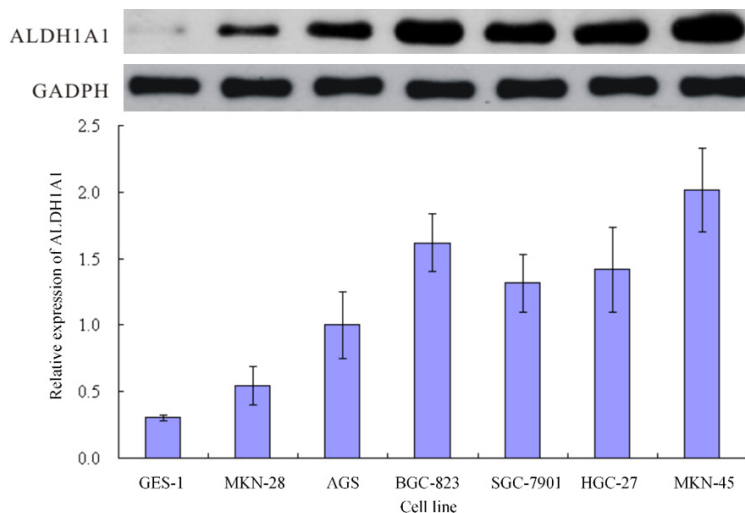


Figure 2. The expression of ALDH1A1 in 6 gastric cancer cell strains detected by RT-PCR and Western blot.

Statistical analysis

Means and standard deviations (SD) were used to summarize continuous variables. To determine the differences among groups, independent t test and one-way ANOVA was used where appropriate. A 2-sided *P* value of <0.05 was considered to be statistically significant. All analyses were performed using SPSS software, version 17.0.

Results

Expression level of the ALDH1A1

The expression level of the ALDH1A1 in gastric cancer tissue (75.0%, 60/80) was higher than para-carcinoma tissue (38.8%, 31/80) (*P* < 0.05) (**Figure 1**). Compared to the immortalized gastric mucosa epithelial cell line GES-1, we

found that the expression of ALDH1A1 in gastric cancer cell line MKN-45 was the highest detected by RT-PCR and Western blot test (**Figure 2**) and suitable as the cell line of follow-up experiments. The ALDH1A1 mRNA and protein expression of shRNA-ALDH1A1 expression vector were decreased by RT-PCR and Western blot detection, which indicated the success of model (**Figure 3**).

Effect of ALDH1A1 on the viability of gastric carcinoma cell and cell cycle

Compared to the control group, the gastric cancer cell vitality significantly decreased in the siRNA-ALDH1A1 after transfection (*P* < 0.01) in **Figure 4**. As shown in **Table 1**, the expression of ALDH1A1 in gastric cancer cell decreased from down from 54.28% to 33.46% in cell cycle G1 phase, and from 33.29% to 22.15% in S phase (both *P* < 0.01).

Effect of ALDH1A1 on migration and invasion in gastric carcinoma cells

The cell migration distance in the ALDH1A1 transfection group (**Figure 5A**) was longer than that in the control group (**Figure 5B**) (42.10 vs. 22.38, *P* < 0.01). The number of gastric cancer cells through the inferior vena in ALDH1A1 transfection group and was more than that in the control group (99.29 vs. 59.37, *P* < 0.05) in **Figure 5C** and **5D**.

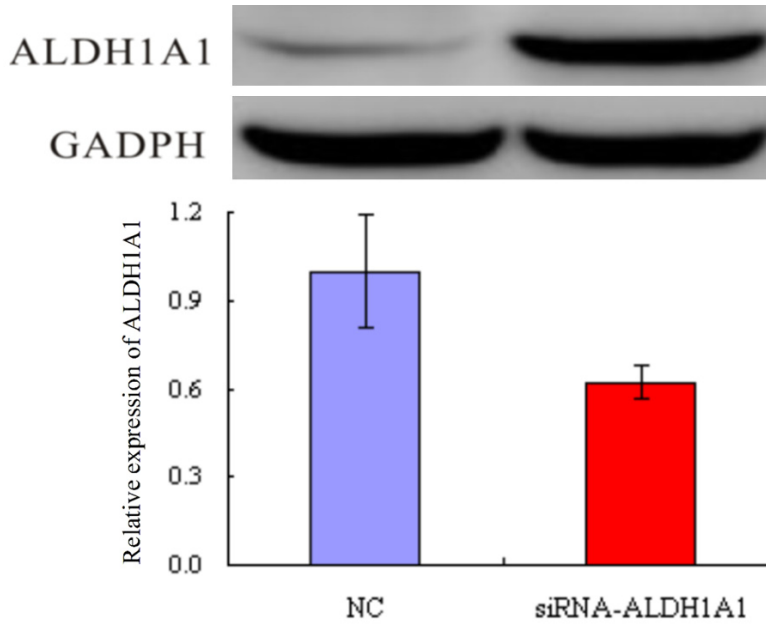


Figure 3. The transfection of ALDH1A1 in gastric cancer cells MKN-45 detected by RT-PCR and Western blot.

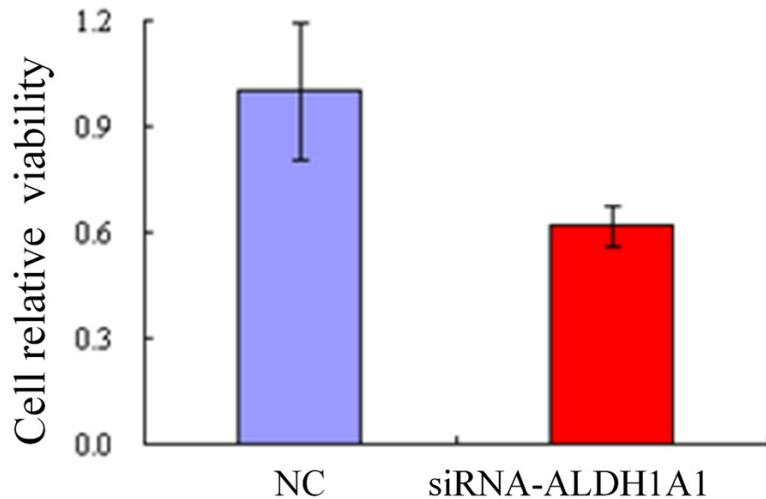


Figure 4. Effect of ALDH1A1 on the viability of gastric carcinoma cell.

Table 1. Effect of ALDH1A1 on gastric cancer cell cycle

Group	G1 (%)	S (%)	G2 (%)
NC	54.28±7.63	33.29±4.21	12.43±1.26
siRNA-ALDH1A1	33.46±5.19	22.15±2.38	44.39±4.05

Effect of ALDH1A1 on the expression of related proteins

The siRNA-ALDH1A1 significantly inhibited the expression of Wnt1, Wnt2 and β-catenin in the

gastric cells (**Figure 6A**), the expression of CyclinD1 and PCNA (**Figure 6B**) and the expression of MMP-2 & MMP-9 (**Figure 6C**) compared to the control group (all $P < 0.01$). ALDH1A1 also up-regulated the expression of E-cadherin and down-regulate the expression of N-cadherin in the gastric cells as shown in **Figure 6D** ($P < 0.01$).

Discussion

ALDH1A1 was highly expressed in a variety of carcinomas, including the gastric carcinoma tissue [9, 10]. The study by Marcato et al. [2] found that the forming ability of breast cancer cells transplanted tumor with high activity of ALDH was significantly enhanced [2]. The abilities of cells colony formation, migration and invasion in vitro of ALDH-positive pancreatic cancer, prostate cancer and bladder cancer, migration were greatly improved [3-5]. Charafe et al. Separated cancer stem cells from three breast cancer cell lines SUM149, MARY-X and IBC, finding that the ALDH1A1 activity was high, and in vivo experiments confirmed that invasion and metastasis abilities of ALDH1A1-positive cancer cells were significantly higher than ALDH1A1-negative cancer cells [13]. Moreb et al. used siRNA to siRNA the ALDH expression of lung cancer cells, which could inhibits proliferation and migration capacities of cancer cells [14]. The study also found 75 up-regulated genes and 26 down-regulated genes by the gene chip technology. These genes were related to the protein connection, signal transduction and cell migration and adhesion, etc. The ovarian cancer cells

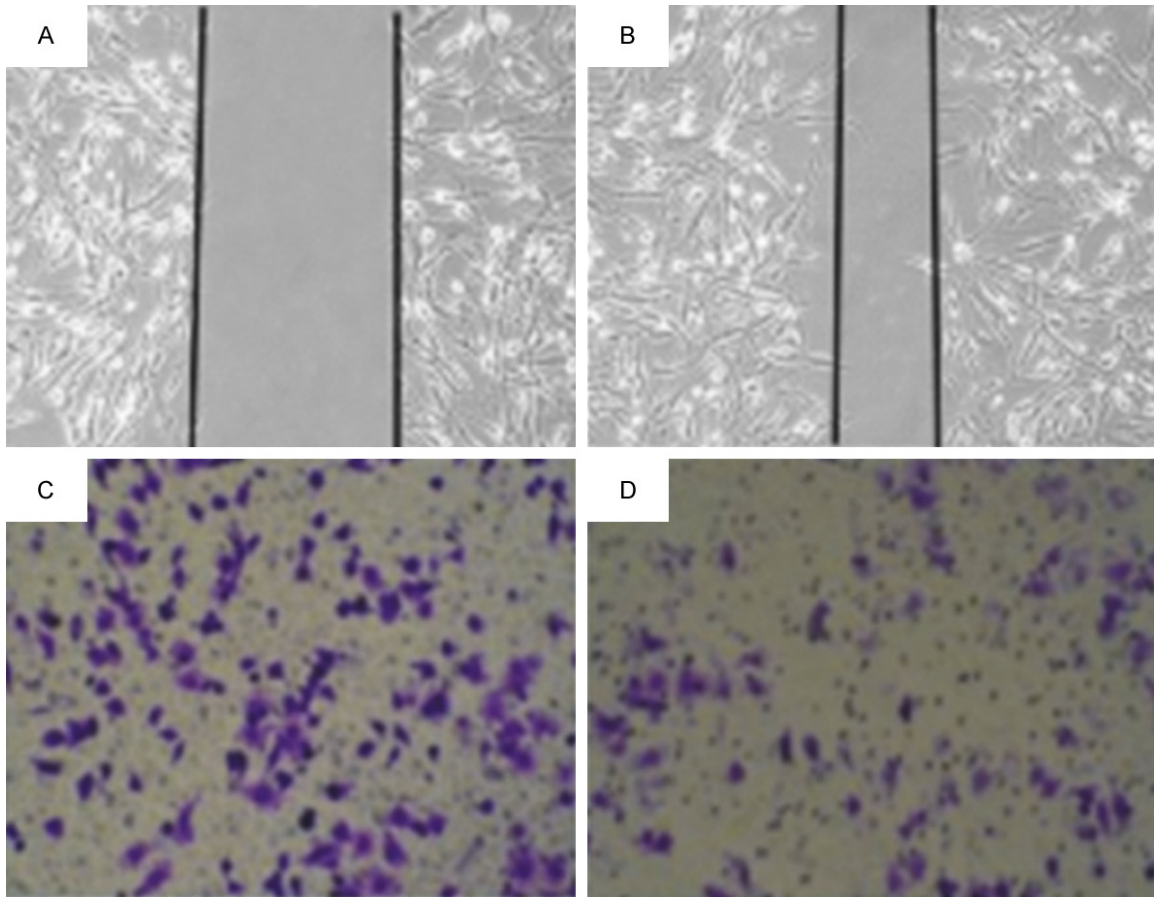


Figure 5. Effect of ALDH1A1 on migration and invasion in gastric carcinoma cell. A: The gastric issue on migration; B: The control group on migration; C: The gastric issue on invasion; D: The control group on invasion.

after ALDH1A1 silence were injected into nude mice, which significantly inhibited tumor growth compared with single chemotherapy [11]. It indicated that ALDH1A1 expression of tumor cells was closely associated with tumor proliferation, migration and invasion, which could be significantly inhibited by interfering the expression of ALDH1A1. Our study confirmed the higher expression of ALDH1A1 in gastric cancer tissues than in adjacent tissues, which was consistent with the studies by Li [9] and Wu [10]. We also found that siRNA-ALDH1A1 was able to inhibit the proliferation, migration and invasion of gastric cancer cells, which was related to Wnt/ β -catenin signaling pathway.

The Wnt gene was discovered by Nusse et al. in the process of inducing breast cancer of mice by HPV in 1982. The Wnt signaling pathway was a class of highly conserved signal transduction pathway in multicellular eukaryotes, closely related to the tumor occurrence and develop-

ment. The study by Katoh et al. confirmed that the expressions of the mRNA of Wnt1 and Wnt2b were increased in gastric cancer tissues [15]. Cheng et al. found that the over expression of Wnt2 protein and the abnormal positioning of the nuclear and plasm of β -catenin protein occurred in gastric cancer tissues [16]. The study by Kurayoshi et al. showed that the Wnt5a mRNA was over expressed in gastric cancer tissues and was related to the invasion and metastasis of cancer cells, which suggested the Wnt signaling pathway was closely related to the occurrence and development of the gastric cancer [17]. Mazieres et al. further inhibited the Wnt-2 protein of human malignant glioma cells by siRNA interference technology and monoclonal anti Wnt-2 antibodies, leading to the inhibition of tumor cells proliferation and the induction of cell apoptosis [18]. ALDH1A1 could be immune coprecipitated with β -catenin, and gene knockout of β -Catenin would lower the expression of ALDH1A1 and inhibit the

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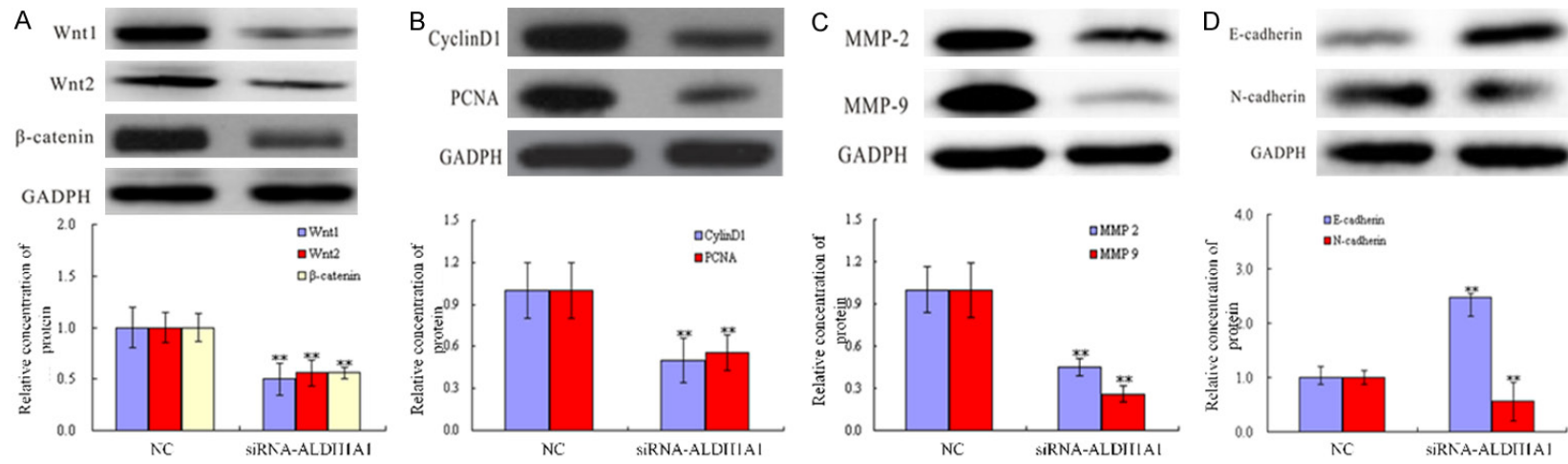


Figure 6. Effect of ALDH1A1 on the expression of related proteins compared to the control group. A. In Wnt/ β -catenin signaling pathway; B. CyclinD1 and PCNA in gastric cancer cells; C. MMPs; D. CyclinD1 and PCNA. Compared to control group, $**P < 0.01$.

growth and peritoneal metastasis of the ovarian transplantation tumor [19]. The expression of ALDH1A1 and CyclinD1 in ovarian cancer cells could be down-regulated by inhibiting the Wnt/ β -catenin signaling pathway, resulting in the inhibition of tumor cells proliferation and the induction of cell apoptosis [20]. In the cells of esophageal squamous cell carcinoma with high activity of ALDH1A1, the expressions of MMP-2 and MMP-9 mRNA were significantly increased while the expression of E-cadherin mRNA was decreased, indicating that highly active ALDH1A1 was able to prompt invasion and metastasis of cancer cells [21]. In the gastric cancer cell line BGC823 and SGC7901 in vitro and in vivo transplantation tumor experiment, the expression of E-cadherin and N-cadherin could be regulated by inhibiting Wnt/ β -catenin signaling pathway [22], suggesting that the expression and activity of ALDH1A1 were closely related to the Wnt signaling pathway, which profoundly affected the proliferation, migration and invasion of tumor cells. Therefore, this study inhibited the proliferation, migration and invasion of gastric cancer cells by interfering the expression of ALDH1A1 in gastric cancer cells, and down-regulated the expression of Wnt1 Wnt2 and β -Catenin, further influencing the expression of downstream target genes.

CyclinD1 is a cycling closely associated with the G1 phase of the cell cycle. It has been reported that CyclinD1 was related to the proliferation of tumor cells [10, 11]. The study by Farago et al. revealed that the Wnt pathway was abnormally activated in breast cancer cell lines, with β -catenin accumulating in the cytoplasm, the expression of CyclinD1 increased, and the proliferation of tumor cells accelerating [23]. The study by Saikawa et al. suggested CyclinD1 was over expressed in the gastric cancer, and the growth of cancer cells was significantly inhibited when interfered by antisense oligonucleotide probes [24]. Cell proliferation antigen (PCNA) is the accessory protein of DNA compound enzyme, which is related to the tumor cell proliferation. PCNA level cyclically changes accompanied with the cell proliferation, starting to express at phase G1 and reaching to the peak at phase S. Therefore, our study interfered ALDH1A1 in gastric cancer cells to down regulate CyclinD1 and PCNA expressions, leading to the block of the cell cycle at phase G1 and the inhibition of cancer cell proliferation.

The extracellular matrix degradation is a necessary step of tumor invasion and metastasis. MMPs are the most important group of proteases to degrade the extracellular matrix, which play an important role in tumor invasion and metastasis. MMP-2 could either degrade the gelatin type IV collagen in the extracellular matrix which would help tumor cells infiltrate to the surrounding parts along the damaged basement membrane, or promote tumor invasion and metastasis through neonatal blood capillaries, which is the sign of the invasion and metastasis of malignant tumors [25]. Tumors with MMP-2 gene amplification and overexpression have high aggressiveness and high degree of malignancy [26]. MMP-9 is a proteolytic enzyme secreted by a variety of cells, and it is the enzyme with largest molecular weight among MMPs which could degrade the extracellular matrix and basement membrane, thus increasing exercise capacity of cells and promoting tumor proliferation and metastasis. MMPs play a crucial role on the invasion of hepatoma cells. Yang et al. found that MMP-9 was significantly over expressed in gastric cancer tissues, and MMP-9 expression in patients with distant metastasis was significantly higher than that in patients without distant metastasis [27]. Meta-analysis of clinical studies indicated that the high expression of MMP-9 contributed to the increase of the invasion of gastric cancer tissues, and affected the prognosis of patients with gastric cancer [28]. Therefore, our study interfered ALDH1A1 of gastric cancer cells to down regulate MMP-2 and MMP-9, thus inhibiting cancer cell migration and invasion.

Epithelial cells-mesenchymal transition (EMT) was first confirmed in the process of embryonic development, and there are increasing evidence demonstrating that EMT plays a significant role in the tumor occurrence, local invasion, metastasis and spread through the circulatory system, which is the characteristic of tumor cells with strongest invasive ability. It could be considered to be the start of the invasion and metastasis when EMT occurred in malignant cells [29, 30]. The symbolic characteristics of EMT is the down-regulation of the expression of intercellular adhesion molecule E-cadherin, and the up-regulation of a series of mesenchymal markers, including the N-cadherin, vimentin and fibronectin [31, 32]. Sun et al. detected the expression of E-cadherin protein of 58 cases of gastric cancer tissues,

40 cases of adjacent tissues and 42 cases of non-cancerous atrophic gastritis by immunohistochemistry technology, finding that the expression of E-cadherin protein in gastric cancer tissues was significantly lower than that in adjacent tissues and non-cancerous atrophic gastritis tissues [33]. Low expression of E-cadherin and high expression of N-cadherin were closely related to gastric cancer invasion and metastasis [34]. Therefore, our study interfered ALDH1A1 of gastric cancer cells to up-regulate the expression of E-cadherin and down-regulate the expression of N-cadherin, thus inhibiting the invasion and metastasis of cancer cells.

In conclusion, interfering ALDH1A1 expression could significantly inhibit the proliferation, migration, invasion and metastasis of MKN-45, and block the cell cycle in phase G1, which were achieved by down-regulating CyclinD1, PCNA, MMP-2, MMP-9 and N-cadherin expression, up-regulating the expression of E-cadherin, and reduce the expressions of Wnt1, Wnt2 and β -catenin.

Disclosure of conflict of interest

None.

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