Aldehyde dehydrogenase 3A1 is robustly upregulated in gastric cancer stem-like cells and associated with tumorigenesis

DI WU[1], YI-PING MOU[1,2], KE CHEN[1], JIA-QIN CAI[3], YU-CHENG ZHOU[2], YU PAN[1], XIAO-WU XU[2], WEI ZHOU[1], JIA-QI GAO[1], DING-WEI CHEN[1] and REN-CHAO ZHANG[2]

[1]Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310016; [2]Department of General Surgery, Zhejiang Provincial People's Hospital, Wenzhou Medical University, Hangzhou, Zhejiang 310014; [3]School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310029, P.R. China

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Abstract. Enhanced aldehyde dehydrogenase (ALDH) activity has been shown to serve as a hallmark for cancer stem cells (CSCs). Recent evidence suggests that its role as a stem cell-related marker has come down to the specific isoform. However, little is known about the specific ALDH isoform contributing to aldefluor activity in gastric cancer. In this study, we isolated ALDH^{bright} cells from 2 human gastric cancer cell lines MKN-45 and SGC-7901 by using an Aldefluor assay and found elevated self-renewal, differentiation and tumorigenicity, as demonstration of stemness characteristics. We also found that ALDH^{bright} cells expressed decreased levels of E-cadherin but increased levels of Snail and Vimentin, indication of an epithelial-mesenchymal transition (EMT) phenotype which may be responsible for the enhanced metastatic potential. Since further research and prognostic application based on ALDH prevalence require the quantification of the specific ALDH isoform, we characterized the expression of all 19 ALDH isoforms in the sorted gastric cancer cell lines by quantitative real-time polymerase chain reaction (qRT-PCR). Compared with the non-stem counterparts, robust upregulation of ALDH-3A1 was observed in these gastric cancer stem-like cells. Furthermore, we performed immunohistological analysis on 93 fixed patient gastric tumor samples and found that ALDH-3A1 expression correlated well with gastric cancer dysplasia and grades, differentiation, lymph node metastasis and cancer stage. Our data, therefore, provide strong evidence that ALDH-3A1 is a novel gastric cancer stem cell related marker with potential prognostic values and demonstrate a clear association between ALDH-3A1 prevalence and gastric cancer progression.

Correspondence to: Professor Yi-Ping Mou, Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, 3 East Qingchun Road, Hangzhou, Zhejiang 310016, P.R. China
E-mail: mouyiping2002@163.com

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Introduction

Gastric carcinoma is a highly aggressive tumor with approximately 50% of cases occurring in Eastern Asia, mainly China (1,2). Although the current operation techniques, chemo- and radio therapies are continuously improving, the 5-year survival rate remains low due to the highly invasive and metastatic properties of gastric carcinoma. Therefore, understanding the crucial events underlying gastric tumorigenesis and progression is urgently needed, which could pave the way for the future anti-anticancer drug discovery and efficacious therapeutic strategies.

It is well known that tumors are composed of heterogeneous cell populations (2-5), which are inconsistent in tumor-propagating ability. Cancer stem cells, also referred to as tumor initiating cells (TICs), which exhibit the self-renewal capacity and highly tumorigenic ability are responsible for tumor invasion, relapse and therapy resistance (6-9). Increasing evidence has suggested that high aldehyde dehydrogenase activity can be used to characterize cells with CSC properties (10-22). The human ALDH enzymes are a family comprised of 19 isoforms (23,24), mainly functioning in oxidizing aldehydes to their corresponding carboxylic acid and converting retinol to retinoic acid (RA) which would activate the RA signaling pathway. Among the various ALDH isoforms, ALDH-1A1 is the most extensively investigated isoform and has always been considered to be the cause of aldefluor activity (18). However, recent studies have shown that not all tumors exhibit the correlation between ALDH-1A1 prevalence and tumor progression, therapeutic resistance or prognosis estimation, that is, ALDH activity is not necessarily ALDH-1A1 specific, but could be attributed to other ALDH isoforms (17,25-30). Therefore, identifying the individual responsible ALDH isoforms in each cancer is of vital importance and can be the foundation of the following research.

ALDH^{bright} gastric cancer cells have recently been demonstrated to possess certain CSC properties (31-33). However, the existing research all regarded ALDH-1 as being responsible for aldefluor activity in gastric cancer, although ALDH-1

families contain 6 members (23,24,34). Few reports identified the specific isoforms responsible for ALDH activity in gastric carcinoma. We therefore investigated the expression of 19 human ALDH isoforms in $ALDH^{bright}$ gastric cancer cells which have been proved possessing certain stemness properties. Our study shows that ALDH-3A1, but not ALDH-1A1 is robustly upregulated in gastric cancer stem-like cells. Moreover, high levels of ALDH-3A1 expression associates with poorly differentiation degree in gastric cancer tissue which suggests that certain level of dedifferentiation may have occurred. Collectively, our research reveals that the aldefluor activity in gastric cancer stem-like cells is primarily due to ALDH-3A1 and its expression correlates well with gastric cancer progression.

Materials and methods

Cell lines and culture conditions. The human gastric cancer cell lines MKN-45 and AGS were obtained from American Type Culture Collection. The other four human gastric cancer cell lines SGC-7901, BGC-823, MGC-803 and HGC-27 were obtained from Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences. MKN-45, SGC-7901, BGC-823, MGC-803 were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovin serum (FBS, Invitrogen). HGC-27 and AGC were cultured in Dulbecco's modified Eagle's medium (Gibco) and F-12K (Boster), respectively supplemented with 10% FBS. All cell lines were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. Images of cell lines were taken by using a Nikon Eclipse TS100 inverted microscope.

Patients and clinical samples. Gastric cancer specimens used in this study were obtained from the 93 cases of gastric cancer patients, with written informed consent, who underwent a surgical resection from 2013 to 2014 at Sir Run Run Shaw Hospital. The samples of patients who underwent preoperative radiotherapy or chemotherapy were excluded. The clinical characteristics of the patients are presented in Table III. The study was approved by the Clinical Research Ethics Committee of Sir Run Run Shaw Hospital of Zhejiang University (no. 20150227-13).

Aldefluor assay and FACS isolation of cells. The ALDH activity of tumor cells was evaluated by Aldefluor kit (Stem Cell Technologies) according to the manufacturer's instructions. Briefly, 10^6 harvested gastric cancer cells were resuspended in 500 μl aldefluor buffer containing 2.5 μl ALDH substrate and incubated for 45 min at 37˚C. The specific ALDH inhibitor diethylaminobenzaldehye (DEAB) served as a negative control. For FACS sorting, cells were labeled using Aldefluor kit and the desired populations were sorted by a FACS Aria cell sorter (BD Bioscience).

Tumorsphere formation assay. For sphere formation assay, cells were cultured in 100-mm ultralow attachment plates (Corning) at a density of 2,000 cells/ml in DMEM with nutrient mixture F-12 (Invitrogen), supplemented with 1% N2 supplement (Gibco), 2% B27 supplement (Gibco), 100 ng/ml epidermal growth factor (Pepro Tech), 20 ng/ml basic

Table I. Tumorigenicity of $ALDH^{bright}$ and $ALDH^{low}$ cells from MKN-45 and SGC-7901.

		Cell dose	
Cell line	Subpopulation	$1x10^3$	$5x10^3$
MKN-45	$ALDH^{bright}$	5/6	6/6
	$ALDH^{low}$	0/6	1/6
SGC-7901	$ALDH^{bright}$	6/6	6/6
	$ALDH^{low}$	0/6	2/6

Table II. Generation of lung metastasis of $ALDH^{bright}$ and $ALDH^{low}$ cells from MKN-45 and SGC-7901.

Cell line	Subpopulation	Lung metastasis (yes/no)
MKN-45	$ALDH^{bright}$	4/2
	$ALDH^{low}$	0/6
SGC-7901	$ALDH^{bright}$	5/1
	$ALDH^{low}$	1/5

Table III. Relationship between ALDH-3A1 and clinicopathological charateristics of gastric cancer.

		Expression of ALDH-3A1		
Clinical factors	Nos.	High	Low	P-value
Age (years)				
<60	47	29	18	0.527
>60	46	25	21	
Gender				
Male	63	38	25	0.237
Female	30	15	15	
T stage				
Tis, T1	1	1	0	0.452
T2	16	8	8	
T3	69	38	31	
T4	7	6	1	
Lymph node metastasis				
Presence	31	23	8	0.015
Absence	62	30	32	
Tumor stage				
I	12	7	5	0.011
II	51	22	29	
III	20	17	3	
IV	10	7	3	
Differentiation				
Well	12	5	7	<0.001
Moderate	40	15	25	
Poor	41	33	8	

fibroblast growth factor (Pepro Tech) at 37˚C in humidified air 5% CO_2. To passage tumorspheres, spheres cultured for 7 days were collected, disaggregated with 0.05% trypsin/EDTA (Solarbio), sieved through a 40-μm filter and replated as described above.

Colony formation assay. For colony formation analysis, two hundred viable sorted cells were plated in each well of 6-well plates and cultured in RPMI-1640 containing 10% FBS. After incubation for 2 weeks at 37˚C, colonies containing >50 cells were counted with Giemsa staining.

Immunofluorescence confocal microscopy. To analyze cell differentiation, the sorted $ALDH^{bright}$ cells were seeded on glass coverslips and cultured in DMEM containing 10% FBS for 7-10 days. The cells were then fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 1 h, blocked in 10% goat serum in PBS for 1 h and labeled overnight at 4˚C with primary mouse anti-human CK-18 antibodies (Zhongshan Gold Bridge Biotechnology) and mouse anti-human CD44 antibodies (Thermo) respectively. After washing the cells with PBS, the cells were incubated for an additional 1 h at room temperature with goat anti-mouse IgG conjugated Alexa 594 (Invitrogen) and goat anti-mouse IgG conjugated FITC (Invitrogen) respectively. Cell nuclei were counterstained with DAPI. Samples were then observed and photographed under immunofluorenscence confocal microscope (Zeiss).

In vivo tumorigenicity assay. The sorted cells were resuspended in 150 μl of 1:1 PBS/Matrigel (BD Bioscience) and injected subcutaneously into the inguen of 6-week-old NOD/SCID mice with $1x10^3$ or $5x10^3$ cells per mouse. Xenograft tumors were removed at the end of 6^{th} week and measured according to the formula: Tumor volume = (length x $width^2$)/2. A portion of each tumor tissue was fixed in 4% formaldehyde for immunohistochemical (IHC) analysis.

Lung metastasis assay. For generation of lung metastasis, $1x10^6$ sorted $ALDH^{bright}$ and $ALDH^{low}$ were resuspended into 300 μl PBS and injected through the lateral tail vein of 6-week-old NOD/SCID mice. All mice were sacrificed at the 16^{th} week, and the lungs were harvested and examined for tumor nodules.

RNA extraction and qRT-PCR analysis. The total RNA of cells was extracted with RNAiso reagent (Takara). cDNA was synthesized using equivalent amount of total RNA (1 μg) with primers in a 20-μl reverse transcriptase reaction mixture (Takara). The primers were designed and purchased from Invitrogen, the sequences of each primer pair were listed in Tables IV and V. qRT-PCR was performed using LightCycler® 480 Real-Time PCR system. The expression of each gene was calculated using $2^{-\Delta\Delta CT}$ method. Results were normalized against the level of GAPDH. All assays were performed in triplicates and results were plotted as the mean ± SD.

Western blot analysis. Cultured cells were lysed with 200 μl of ice-cold RIPA buffer (Beyotime) containing 1 mM PMSF. Proteins were resolved by SDS-polyarylamide gel and transferred to PVDF membranes (Millipore). The membranes were then blocked with 5% non-fat milk and were then incubated with primary antibodies overnight at 4˚C. The membranes were washed and incubated with appropriate HPR-conjugated secondary antibodies for 1 h at room temperature. Protein

Table IV. Real-time PCR primers used for all 19 human ALDH isoforms and GAPDH.

Genes	Sequences
ALDH1A1	F: 5'-ACTGCTCTCCACGTGGCATCTTTA-3'
	R: 5'-TGCCAACCTCTGTTGATCCTGTGA-3'
ALDH1A2	F: 5'-AGGGCAGTTCTTGCAACCATGGAA-3'
	R: 5'-CACACACTCCAATGGGTTCATGTC-3'
ALDH1A3	F: 5'-ACCTGGAGGTCAAGTTCACCAAGA-3'
	R: 5'-ACGTCGGGCTTATCTCCTTCTTCC-3'
ALDH1B1	F: 5'-TGCTGCAGAGTGTCAGCAT-3'
	R: 5'-GGTGGTAGGGTTGACCGTCG-3'
ALDH1L1	F: 5'-ATCTTTGCTGACTGTGACCT-3'
	R: 5'-GCACCTCTTCTACCACTCTC-3'
ALDH1L2	F: 5'-GCCTGGTCTCGTTACCAAAA-3'
	R: 5'-GCCACTTTCACCTCTTCAGC-3'
ALDH2	F: 5'-CCAACCAGCAGCCCGAGGTC-3'
	R: 5'-AAGGCCTTGTCCCCTTCAGCTACC-3'
ALDH3A1	F: 5'-TGTGTCAAAGGCGCCATGAGCAAG-3'
	R: 5'-GGCGTTCCATTCATTCTTGTGCAG-3'
ALDH3A2	F: 5'-TGCACTTCACGCTCAACTCT-3'
	R: 5'-GACTGGCTGTTGGGAGGATA-3'
ALDH3B1	F: 5'-ACAAGTCAGCCTTCGAGTCGG-3'
	R: 5'-AGCACCACACAGTTCCCTGC-3'
ALDH3B2	F: 5'-ACAGAGAAGGTCCTGGCTGA-3'
	R: 5'-CATGACAATCTTGCCCACAC-3'
ALDH4A1	F: 5'-TGCAGTACCAAGTGTCGCCTTT-3'
	R: 5'-AATCTCCGCTTGGATCACGGTCTT-3'
ALDH5A1	F: 5'-ACCAATTCTTGGTGCAAAGG-3'
	R: 5'-GTTGGTGTCGTTTTCCACCT-3'
ALDH6A1	F: 5'-GGCTCTTTCAACAGCAGTCC-3'
	R: 5'-ATGGAAGCTCCCTCCTTTGT-3'
ALDH7A1	F: 5'-CGAGCCAATAGCAAGAGTCC-3'
	R: 5'-CTTCACCCACACCTTCCACT-3'
ALDH8A1	F: 5'-TGGTGAGCATAGGTGCTCTG-3'
	R: 5'-GTTATCACCGTGGGAAGCAT-3'
ALDH9A1	F: 5'-CACTCATCAACCGACCACAC-3'
	R: 5'-GGACATAACAGGCCCAAAGA-3'
ALDH16A1	F: 5'-GCTCCTCATCCAGGAGTCTG-3'
	R: 5'-AAGGTTGGGGGATAGAATGG-3'
ALDH18A1	F: 5'-CTGAGTATGGGGACCTGGAA-3'
	R: 5'-GCGGTAACCATCAGAAAAGC-3'

Table V. Real-time PCR primers used for EMT associated transcription factors and stem cell related markers.

Genes	Sequences
Snail	F: 5'-GACCACTATGCCGCGCTCTT-3'
	R: 5'-TCGCTGTAGTTAGGCTTCCGATT-3'
Slug	F: 5'-AGCGAACTGGACACACATAC-3'
	R: 5'-TCTAGACTGGGCATCGCAG-3'
Twist-1	F: 5'-CACTGAAAGGAAAGGCATCA-3'
	R: 5'-GGCCAGTTTGATCCCAGTAT-3'
Zeb-1	F: 5'-CGAGTCAGATGCAGAAAATGAGCAA-3'
	R: 5'-ACCCAGACTGCGTCACATGTCTT-3'
Zeb-2	F: 5'-GAGTTGATGCCTCGGCTATTGC-3'
	R: 5'-CTGGACATTGAGCTGCTTCGATC-3'
E-cadherin	F: 5'-TACACTGCCCAGGAGCCAGA-3'
	R: 5'-TGGCACCAGTGTCCGGATTA-3'
Vimentin	F: 5'-TGAGTACCGGAGACAGGTGCAG-3'
	R: 5'-TAGCAGCTTCAACGGCAAAGTTC-3'
OCT-4	F: 5'-GCAGCGACTATGCACAACGA-3'
	R: 5'-CCAGAGTGGTGACGGAGACA-3'
NANOG	F: 5'-GACTTGACCACCGAACC- CAT-3'
	R: 5'-CTGGATGTTCTGGGTCTGGT-3'
BMI-1	F: 5'-TCGTTCTTGTTATTACGCTGTTTT-3'
	R: 5'-CGGTAGTACCCGCTTTTAGGC-3'
GAPDH	F: 5'-GGTGTGAACCATGAGAAGTATG-3'
	R: 5'-GATGGCATGGACTGTGGTCAT-3'
Snail	F: 5'-GACCACTATGCCGCGCTCTT-3'
	R: 5'-TCGCTGTAGTTAGGCTTCCGATT-3'
Slug	F: 5'-AGCGAACTGGACACACATAC-3'
	R: 5'-TCTAGACTGGGCATCGCAG-3'
Twist-1	F: 5'-CACTGAAAGGAAAGGCATCA-3'
	R: 5'-GGCCAGTTTGATCCCAGTAT-3'
Zeb-1	F: 5'-CGAGTCAGATGCAGAAAATGAGCAA-3'
	R: 5'-ACCCAGACTGCGTCACATGTCTT-3'
Zeb-2	F: 5'-GAGTTGATGCCTCGGCTATTGC-3'
	R: 5'-CTGGACATTGAGCTGCTTCGATC-3'
E-cadherin	F: 5'-TACACTGCCCAGGAGCCAGA-3'
	R: 5'-TGGCACCAGTGTCCGGATTA-3'
Vimentin	F: 5'-TGAGTACCGGAGACAGGTGCAG-3'
	R: 5'-TAGCAGCTTCAACGGCAAAGTTC-3'
OCT-4	F: 5'-GCAGCGACTATGCACAACGA-3'
	R: 5'-CCAGAGTGGTGACGGAGACA-3'

bands were detected by using Pierce ECL Western blotting substrate (Thermo). GAPDH or TUBLIN were used as a loading control. The primary antibodies used for western blot analyses were as follows: NANOG (Abcam), OCT-4 (Abcam), BMI-1 (Abcam), ALDH-1A1 (BD Bioscience), ALDH-3A1 (Santa Cruz Biotechnology), GAPDH (Abcam), TUBLIN (Cwbiotech).

TMA and image analysis. TMAs were either constructed by author (Key Laboratory of Biotherapy of Zhejing University) or purchased from Alenabio Biotech Co.. Staining of tissue microarray slides was carried out according to the manufacturer's protocol. Briefly, IHC staining was performed on 10% phosphate-buffered formalin fixed and paraffin-embedded gastric cancer sections (4 μm) using xylene. The slides were hydrated by a graded series of ethanol washes and incubated in 0.3% H_2O_2. After incubation with blocking solution for 30 min at room temperature, the slides were incubated with primary antibodies at 4˚C overnight. The secondary antibodies were added for incubation at 37˚C. TMA slides were scanned by using Aperio Slide Scanner and analyzed by Image Scope software (Aperio). The IHC staining was scored independently by two pathologists blinded to the clinical data as follows: Score = percentage of immunoreactive cells (0, <10%; 1, 11-25%; 2, 26-50%; 3, 51-75%; 4, >75%) x mean stain intensity (0-3).

Statistical analysis. Statistical analysis was performed using SPASS statistical analysis software Version 17.0 (SPSS). Student's t-test was used to examine the statistical significance when two groups were compared. To determine differences among 3 groups, an ANOVA analysis was performed. P-value <0.05 was regarded as statistically significant.

Results

$ALDH^{bright}$ gastric cancer cells exibit cancer stem-like cell properties. We first examined the proportion of $ALDH^{bright}$ cells in the six human gastric cancer cell lines using Aldefluor kit and found that each of the 6 gastric cancer cell lines (MKN-45, SGC-7901, HGC-27, AGS, BGC-823, MGC-803) contained ALDH-positive cells (Fig. 1A). Compared with the DEAB-treated control groups, high ALDH activity was detected in 22.1% of the MKN-45 cells, 18.3% of the SGC-7901 cells, 1.40% of the HGC-27 cells, 1.25% of the MGC-803 cells, 1.24% of the AGS cells, 0.36% of the BGC-823 cells (Fig. 1D). We therefore chose MKN-45 and SGC-7901 for the follow-up research taking advantage that both cell lines contained prominent $ALDH^{bright}$ subpopulations (indicated by the bright green fluorescence), making the ALDH activity based separation more efficient and convincing (Fig. 1B and C). We present here that $ALDH^{bright}$ cells isolated from the 2 gastric cancer cell lines showed higher capabilities of tumorsphere formation compared with $ALDH^{low}$ cells. These spheres could be passaged for at least 3 consecutive generations with an increasing forming efficiency, while the $ALDH^{low}$ cells did not form typical tumorspheres, only a few loose cell aggregates (Fig. 2A). The number of colonies formed by $ALDH^{bright}$ MKN-45 cells and $ALDH^{bright}$ SGC-7901 cells were higher than those formed by $ALDH^{low}$ cells (138 versus 36, 150 versus 49, respectively) (Fig. 2B). The $ALDH^{bright}$ cells from MKN-45 and SGC-7901 also showed multi-potent capacity of differentiation by expressing decreased level of CD44 but increased level of CK-18 after induction of differentiation (Fig. 2C). $ALDH^{bright}$ MKN-45 and SGC-7901 were also found to express higher levels of stem cell-related markers including OCT-4,

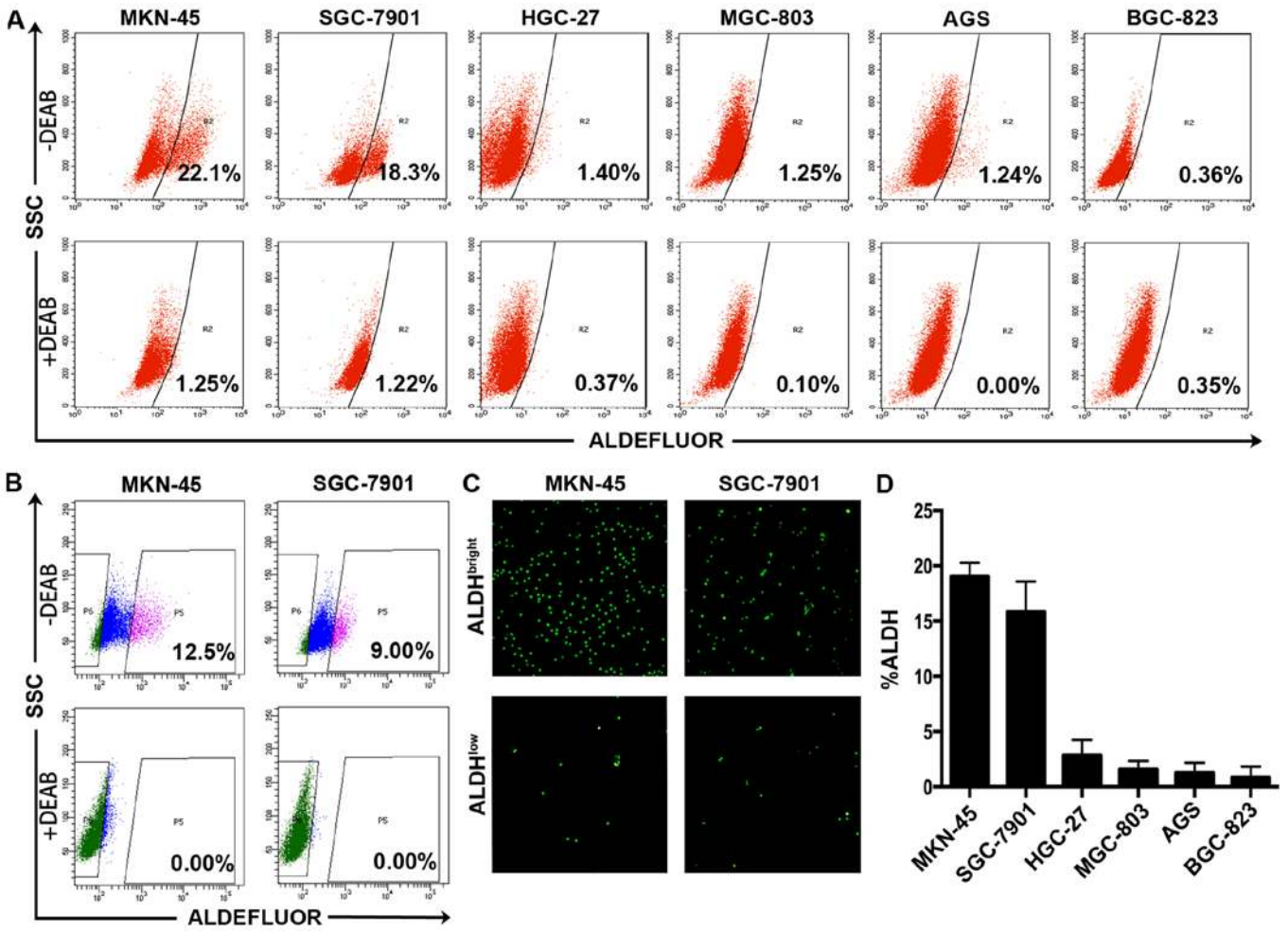

Figure 1. ALDH expression in human gastric cancer cell lines. (A) ALDH^{bright} cells in 6 human gastric cancer cell lines were analyzed by flow cytometry. DEAB was used as the specific ALDH inhibitor in the negative control groups and the gated cells were defined as ALDH^{bright} cells. (B) ALDH^{bright} and ALDH^{low} cells were isolated from MKN-45 and SGC-7901 cell lines using a FACS Aria cell sorter. (C) Gastric cancer cells were stained by using Aldefluor kit and ALDH^{bright} cells (bright green fluorescence) were detected by fluorescent microscopy. (D) Average percentage of ALDH^{bright} cells in six gastric cancer cell lines, determined by Aldefluor assay.

BMI-1, NANOG than ALDH^{low} cells (Fig. 2D). The tumorigenicity assay with isolated ALDH^{bright} and ALDH^{low} gastric cancer cells in NOD/SCID mice showed that ALDH^{bright} MKN-45 and SGC-7901 cells have enhanced tumor initiation capacity as compared to ALDH^{low} cells at each cell dose (10^3, 5/6 versus 0/6, 6/6 versus 0/6; $5x10^3$, 6/6 versus 1/6, 6/6 versus 2/6, respectively) (Fig. 2E and Table I). Also, the volume of ALDH^{bright} cell derived xenografts were larger than those of ALDH^{low} cell-derived (Fig. 2E).

ALDH^{bright} cells display enhanced metastatic ability correlating with a mesenchymal transition phenotype. Accumulating evidence suggests that CSCs are responsible for tumor relapse, we therefore examined the metastatic capabilities and the expression of related molecules in the sorted ALDH^{bright} cells. The ALDH^{bright} cells derived from MKN-45 and SGC-7901 formed lung metastasis in four of six mice, and five of six mice, respectively, whereas few metastasis was formed by ALDH^{low} cells (Fig. 3A and Table II). Gene expression analysis further showed that MKN-45 ALDH^{bright} cells express higher levels of Vimentin and Snail but lower level of E-cadherin, similar results were also obtained in SGC-7901 ALDH^{bright} cells (Fig. 3B), suggesting EMT might be responsible for their enhanced metastatic capabilities.

Specific increase in ALDH-3A1 expression in ALDH^{bright} subpopulations. Since the ALDH isoform responsible for Aldefluor activity may vary depending on cancer type and tissue origin, we characterized the gene expression of all 19 ALDH isoforms between ALDH^{bright} and ALDH^{low} cell populations sorted from MKN-45 and SGC-7901. Our results showed that among all the various human ALDH isoforms, ALDH-3A1 was the most elevated isoform in the sorted MKN-45 and SGC-7901 cells (15.9- and 11.2-fold, respectively) (Fig. 4A and B). In addition, several other isoforms were also found upregulated including ALDH-16A1, ALDH-2, ALDH-3B1, ALDH-4A1, ALDH-3B2 in MKN-45 derived ALDH^{bright} cells, ALDH-18A1, ALDH-2, ALDH-3B2 in SGC-7901 derived ALDH^{bright} cells, the elevated folds of which ranges from 4.4 to 5.8, 4.0 to 4.6, respectively. Notably, no fold change was detected in ALDH-1A1 in ALDH^{bright} MKN-45 and SGC-7901 cells. Moreover, the mRNA levels of

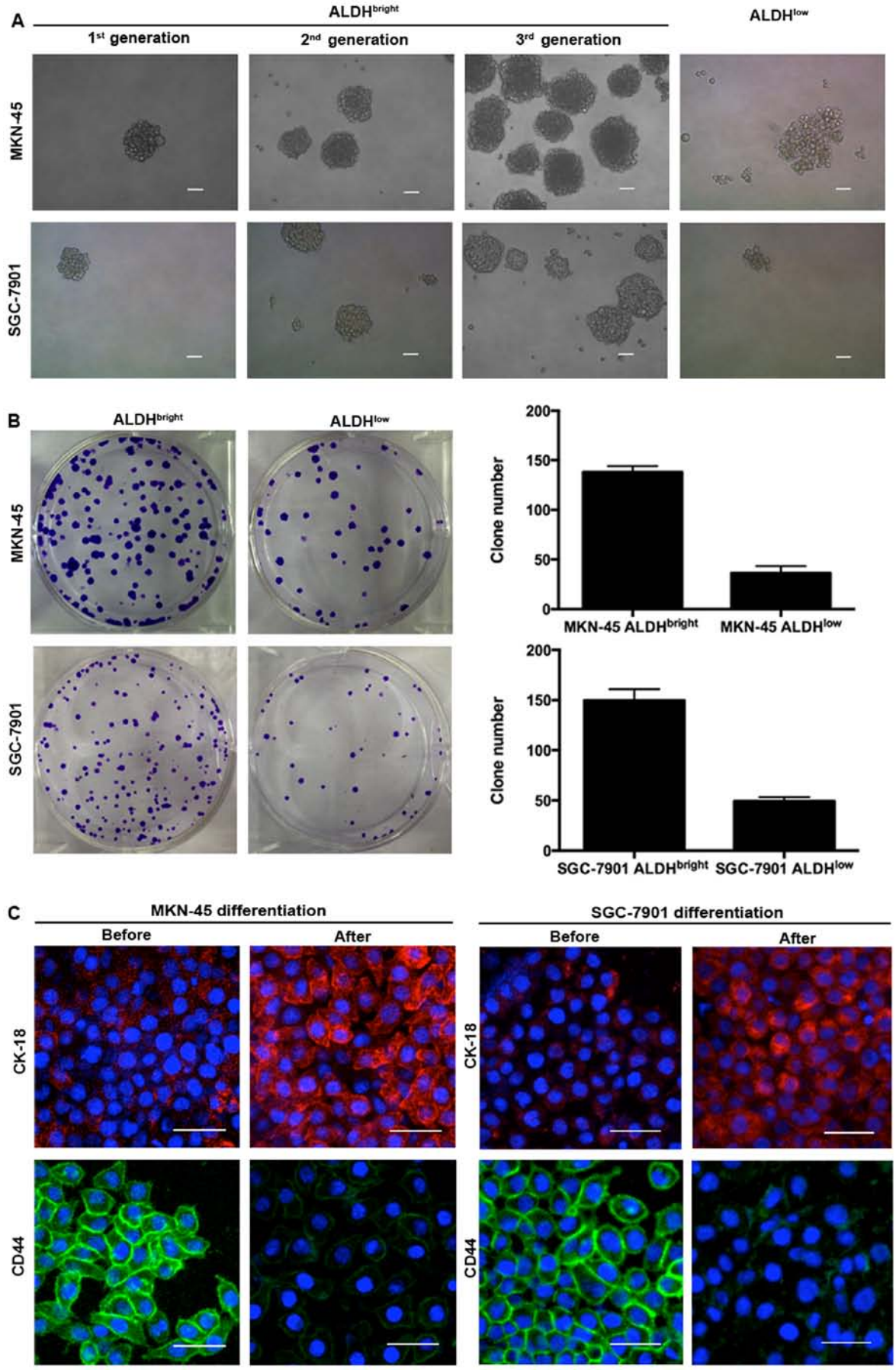

Figure 2. Characterization of ALDH^{bright} cell populations sorted from MKN-45 and SGC-7901 cell lines. (A) Representative images of the primary, secondary and tertiary tumor spheres formed by ALDH^{bright} and ALDH^{low} cells from MKN-45, SGC-7901 are shown. Bar, 50 μm. (B) Representative images and quantitative analysis of colony formation of the sorted ALDH^{bright} and ALDH^{low} cells (mean ± SD). (C) Detection of CD44 and CK-18 expression by immunofluorescence confocal microscopy in MKN-45 derived ALDH^{bright} cells and SGC-7901 derived ALDH^{bright} cells. Bar, 100 μm.

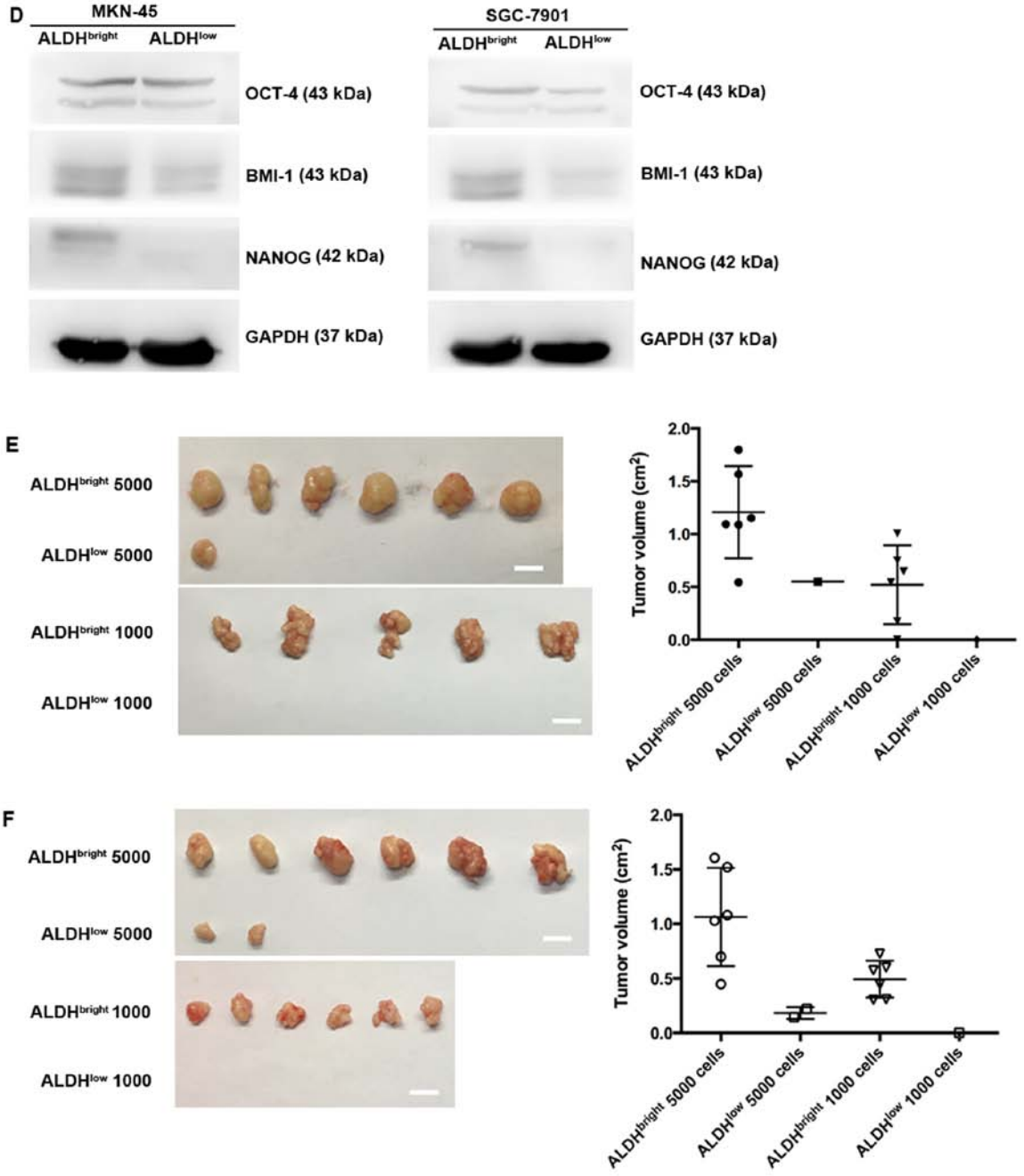

Figure 2. Continued. (D) Western blot analysis of the protein levels of stem cell-related transcription factors OCT-4, NANOG, BMI-1 in ALDH^{bright} and ALDH^{low} subpopulations from MKN-45 and SGC-7901. GAPDH was used as a loading control. Representative images and quantitative analysis of the volumes of xenograft tumors from (E) MKN-45 ALDH^{bright} and ALDH^{low} cells and (F) SGC-7901 ALDH^{bright} and ALDH^{low} cells in NOD/SCID mice.

ALDH-1A1 were undetectable until cycle 35 for two cell lines. The expression of ALDH-3A1 was subsequently confirmed by western blot analysis (Fig. 4C), immunocytochemical (ICC) staining (Fig. 4D) and IHC staining (Fig. 4E).

ALDH-3A1 expression associates with gastric cancer evolution. To understand the possible role of ALDH-3A1 in the development and progression of gastric cancer, we examined the expression of ALDH-3A1 in a set of primary gastric tumors of different severity and metastatic gastric tumors. We were able to show here that ALDH-3A1 could not be detected or was only slightly expressed in the basal layer in normal gastric epithelia, but was detectable at low levels in the gastric dysplastic lesions, then the staining intensity of ALDH-3A1 progressed from weak to strong with increasing grades of dysplasia and carcinoma (Fig. 5B and C). The expression of ALDH-3A1 was associated with histological degrees ($P<0.001$), N stage ($P=0.015$) and cancer stage ($P=0.011$) of gastric cancer (Table III). Considering that CSCs play critical roles in tumor relapse, we subsequently examined the level of ALDH-3A1 in several pairs of local and lymph node metastatic gastric cancer and found higher levels of ALDH-3A1 in metastatic lesions compared with the local carcinomas (Fig. 5D). Collectively, these results indicate that the expression level of ALDH-3A1 correlates with the increasing severity of gastric cancer.

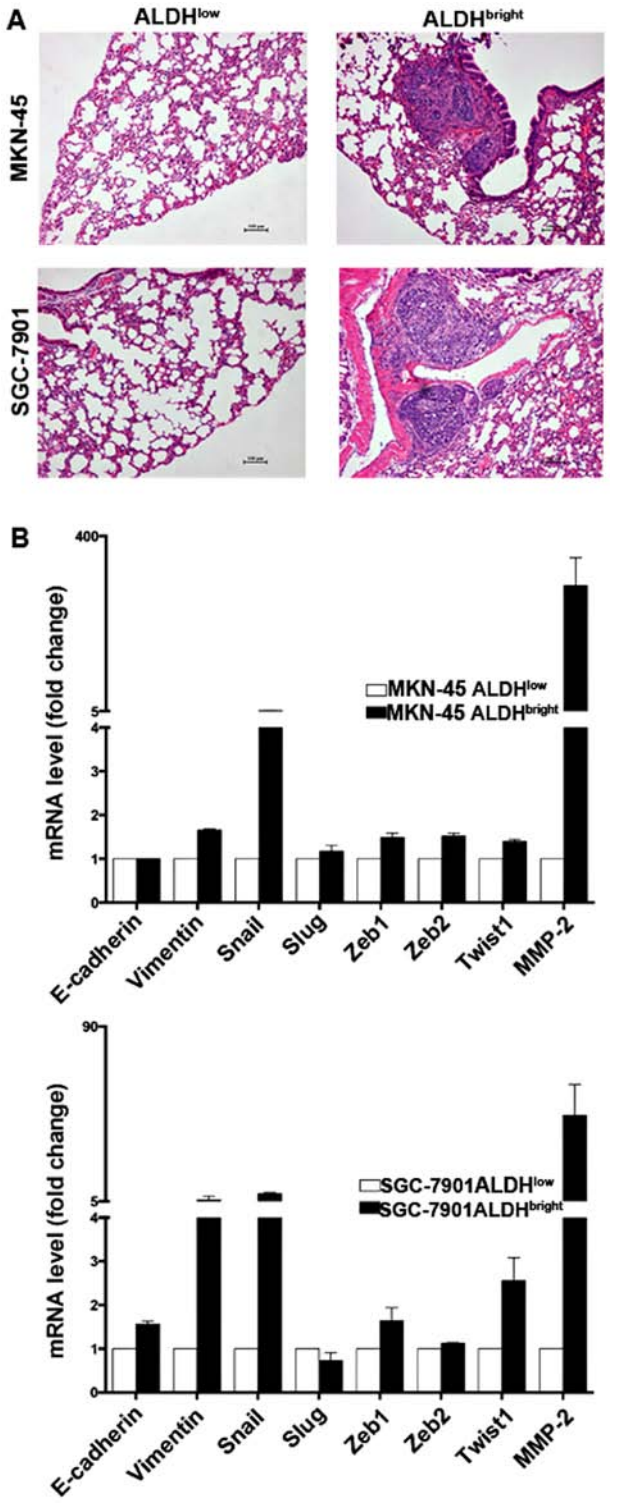

Figure 3. Analysis of metastasis capabilities and the expression of metastatic related molecules of ALDH^{bright} cells. (A) Generation of lung metastasis of MKN-45 ALDH^{bright} cells and SGC-7901 ALDH^{bright} cells in NOD/SCID mice. (B) qRT-PCR analysis of the protein levels of E-cadherin, Vimentin, Snail, Slug, Zeb1, Zeb2, Twist1, MMP-2 in ALDH^{bright} and ALDH^{low} cells, GAPDH was used as an internal control. Corresponding mRNA in ALDH^{bright} cells are shown as a fold change to ALDH^{low} cells (mean ± SD).

Discussion

In this study, we provide strong evidence that ALDH^{bright} gastric cancer cells possess cancer stem-like properties and revealed for the first time that ALDH-3A1 is the highly expressed isoform in gastric cancer stem-like cells. Together with IHC analysis in tissue level, we show that ALDH-3A1-expressing cells play a vital role in the initiation and progression of gastric cancer. We convincingly demonstrate that ALDH-3A1 functions as a pivotal enzyme in regulating gastric cancer stem-like population, ALDH-3A1 prevalence therefore might be used as a novel indicator to predict gastric cancer patient outcome.

The human ALDH enzymes are a family comprised of 19 isoforms, mainly functioning in oxidizing various aldehydes to their corresponding carboxylic acids (27,34). High ALDH activity was originally identified as a marker for hematopoietic stem cells (25,35,36) and is thought to play a vital role in the self-renewal and differentiation of hematopoietic progenitor cells via converting retinal to retinoic acid (37). Recent studies suggest that high levels of ALDH activity characterize a subpopulation of cells with CSC properties in several malignancies (14,19-21,38,39). Moreover, enhanced ALDH activity predicts poor patients outcome in breast cancer (11,22,40-42), lung cancer (15,40), and esophageal squamous cell carcinoma (43), which is consistent with CSCs being responsible for cancer evolution and metastasis. Katsuno *et al* (32) have reported that ALDH^{bright} cells derived from HSC-39 and OCUM-2ML gastric cancer cell lines exhibited certain stem cell traits but little is known about traits of ALDH^{bright} cells in other gastric cancer cell lines. In this study, we isolated ALDH^{bright} and ALDH^{low} subpopulations from 2 different gastric cancer cell lines MKN-45 and SGC-7901. Our data show that these sorted cells have capabilities of self-renewal, multilineage differential potential, high tumorigenicity and express high levels of stemness associated hallmarks, thereby representing a more reliable cancer stem-like cell marker in gastric cancer. We also found that the ALDH^{bright} cells are more invasive and exhibit a phenotype of EMT by expressing increased level of Vimentin and Snail but decreased level of E-cadherin, in agreement with EMT being able to endow tumor cells with stem cell-like properties. We have previously reported that Snail is required for the maintenance of stem cell-like phenotype in pancreatic carcinoma (44), as such, it would be interesting to examine its contributions to gastric cancer stem-like compartment, which might provide novel therapeutic strategies.

With our discoveries that ALDH^{bright} gastric cancer cells exhibit stemness charateristics and may contribute to tumor invasion, we hypothesized that the activity of ALDH in tumor might associate with gastric tumorigenesis and provide clinical prognostic values in gastric cancer patients. Generally, CSCs quantification in clinic requires immunohistological methods for detection of the specific protein expression in fixed tumor tissue. Therefore, the prognostic application by evaluating ALDH prevalence requires detection and quantification of ALDH expression at protein level in gastric tissue, but not activity. Although the Aldefluor assay is able to identify the celluar ALDH activity, it is not practical for clinical evaluation of ALDH at protein level. More importantly, the Aldefluor assay has been proved to be not merely ALDH-1A1-specific. Increasing evidence has demonstrated that the ALDH isoforms responsible for Aldefluor assay may vary depending on the cell origin and tumor types, the reason of which could be attributed to the cross-reactivity between the similar amino acid sequence

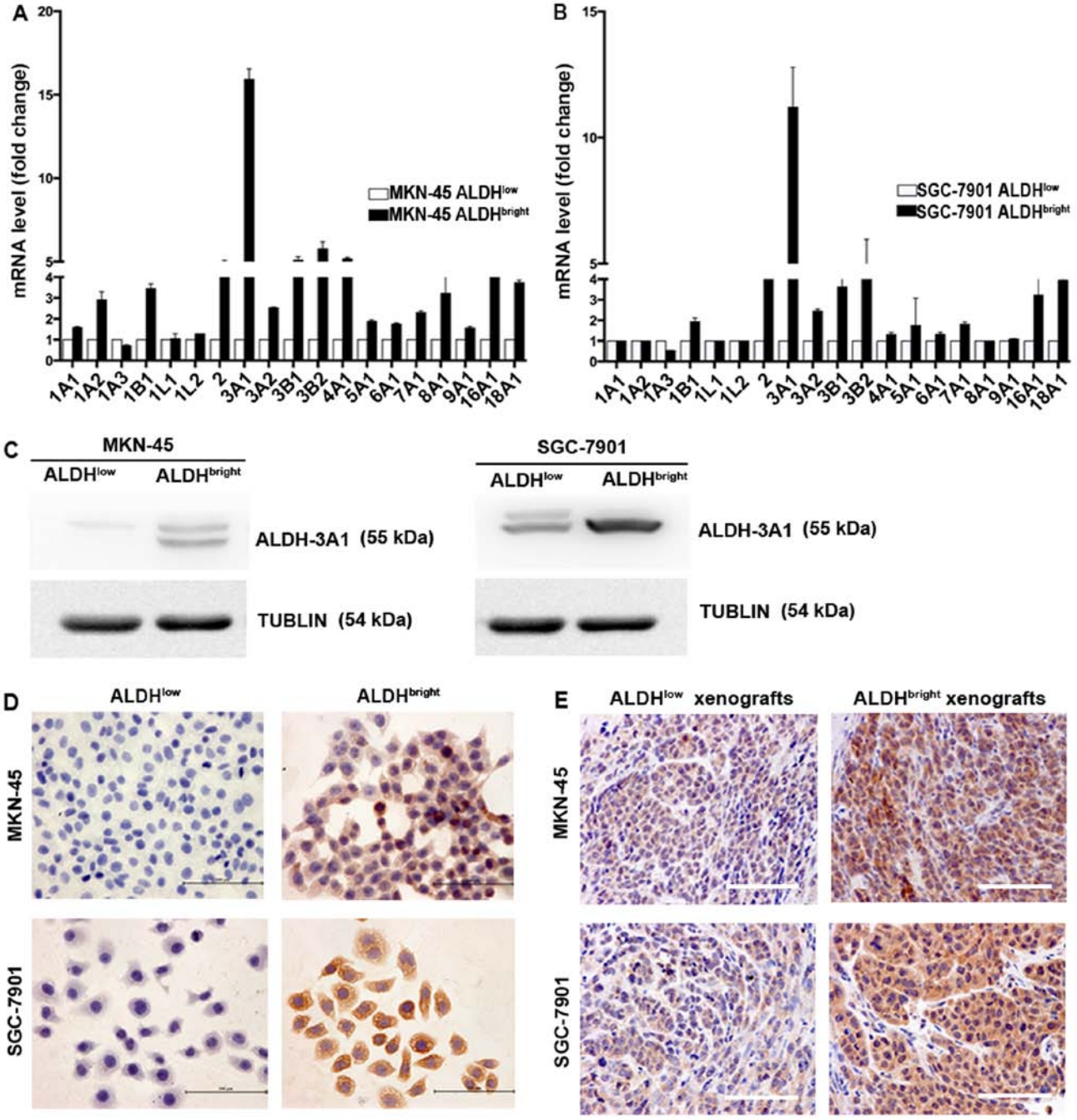

Figure 4. Specific increase in ALDH-3A1 in ALDH^{bright} cells compared with ALDH^{low} cells. (A and B) qRT-PCR was carried out on all 19 ALDH isoform genes for ALDH^{bright} and ALDH^{low} subpopulations from MKN-45, SGC-7901. GAPDH was used as an internal control. Corresponding ALDH mRNA in ALDH^{bright} cells are shown as a fold change to ALDH^{low} cells (mean ± SD). (C) Western blot analysis of the protein levels of ALDH-3A1 in the ALDH^{bright} and ALDH^{low} subpopulations. TUBLIN was used as an internal control. (D) The expression of ALDH-3A1 in tumorspheres formed by ALDH^{bright} and ALDH^{low} cells was measured by ICC analysis. Bar, 100 μm. (E) The expression of ALDH-3A1 in xenograft tumors derived from the sorted MKN-45 and SGC-7901 cells. Bar, 100 μm.

sharing among 19 ALDH isoforms. Van den Hoogen *et al* (45) reported high expression of ALDH-7A1 in both prostate cancer cell lines and malignant tissues. Yan *et al* (46) demonstrated the association of ALDH-3A1 with prostate cancer progression. These findings suggest that more than one of these isoforms could be responsible for the Aldefluor activity. Chen *et al* (25) compared the expression levels of ALDH-1A1 and ALDH-3A1 in colon adenocarcinoma and reported that 98% of the tumor samples were ALDH-1B1 positive. Marcato *et al* (26) characterized the Aldefluor positive cells isolated from breast cancer patient tumor samples and found a better correlation with ALDH-1A3. Similar studies have successively been published and attracted broad attention in defining the responsible ALDH isoforms in different tumors. So far, the specific ALDH isoform responsible for Aldefluor activity in gastric cancer is still unclear. Furthermore, staining with ALDH-1A1 has shown no prognostic impact in gastric cancer patients (33), suggesting that ALDH-1A1 might not be attributed to ALDH activity, thus unable to provide clinical prognostic value. We show here the obvious

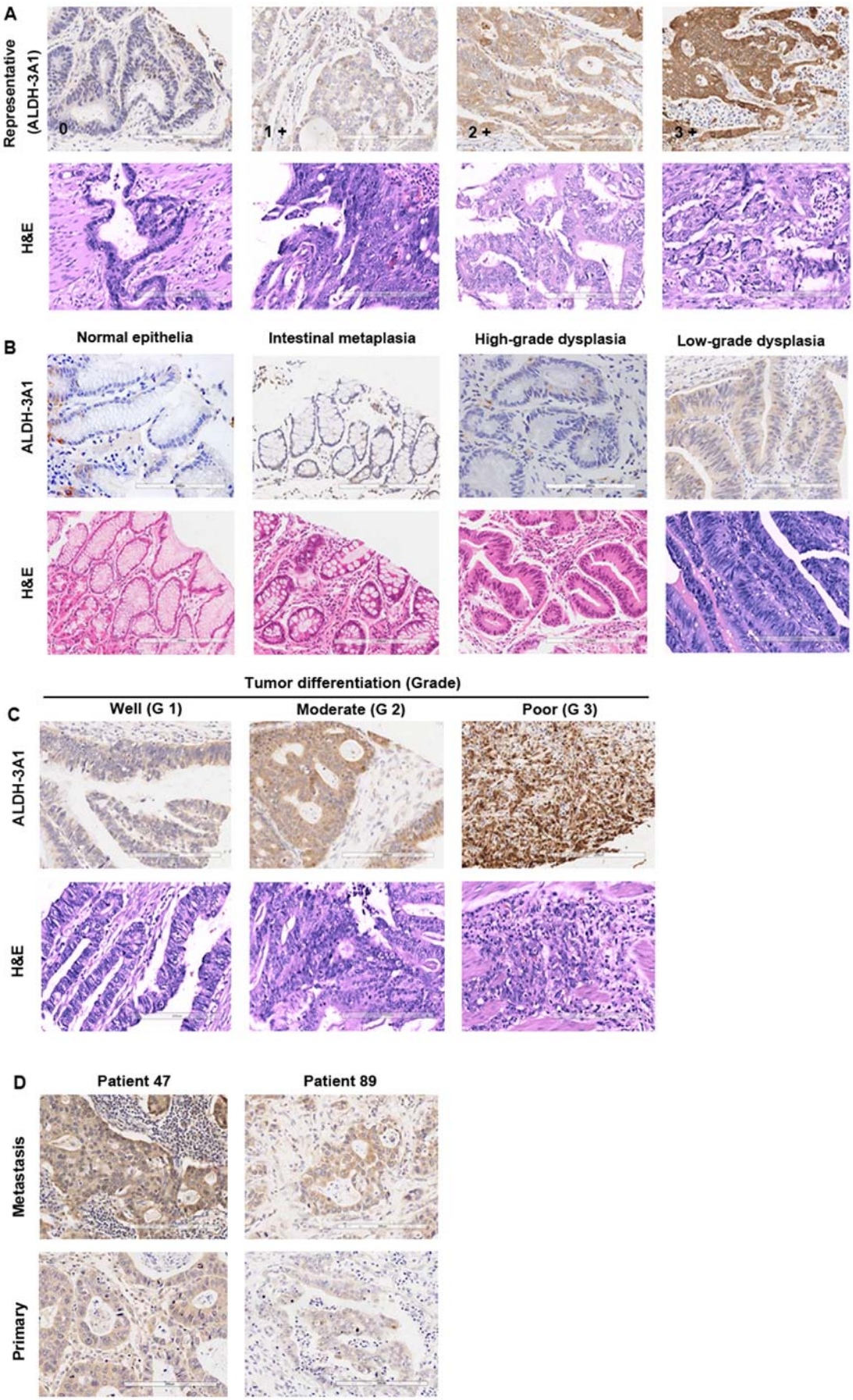

Figure 5. IHC analysis of ALDH-3A1-positive cells in normal gastric mucosa and gastric carcinoma samples with different severity. (A) Representative IHC staining intensity of ALDH-3A1 from patients with gastric adenocarcinoma. In all cases, scores were assigned to a scale of 0-3; 0, no staining; 1+, mild; 2+, moderate; and 3+, strong intensity of staining. (B) Expression for ALDH-3A1 in normal gastric epithelia, gastric dysplastic lesions and gastric epithelia dysplasia. (C) Representative IHC images of ALDH-3A1-positive cells in gastric cancer tissue with different histological grade. (D) Expression for ALDH-3A1 in local gastric carcinoma and corresponding lymph node metastasis lesions. G1, G2 and G3, tumor histological grade I, II and III, respectively.

upregulation of ALDH-3A1 in both MKN-45 and SGC-7901 derived $ALDH^{bright}$ cells compared with $ALDH^{low}$ cells. In the tissue level, the staining intensity of ALDH-3A1 elevates along with increasing severity of gastric cancer, suggesting the expression of ALDH-3A1 may have resulted in certain level of dedifferentiation in which process gastric cancer stem-like cells may have been produced and contributed to gastric tumorigensis. Particularly, enhanced expression of ALDH-3A1 was observed in gastric cancer metastatic lesions compared to the matched primary carcinoma. This fits well with $ALDH^{bright}$ cells possessing increased metastatic capabilities with EMT phenotype. In addition, ALDH-1A1 expression could not be detected in sorted gastric cancer stem-like cells, suggesting ALDH-1A1 might not function in gastric cancer stem cell biology, thereby could not be deemed as a cancer stem cell related marker in gastric cancer. We speculate this possibility could account for the negative follow-up results of applying ALDH-1A1 prevalence to predict gastric cancer patients outcome.

ALDH-3A1 is an important isoform of ALDH family located in the cytoplasm (27) and shows multiple catalysis including serving as a corneal crystalline to protect eyes from UV radiation, contributing to the maintainance of hematopoietic stem cell population, regulating cell proliferation and apoptosis and conducing to the chemotherapeutic drug resistance (47-54). All these functions in particular have been linked to tumor evolution. We demonstrate here that ALDH-3A1 is involved in the process of malignant transformation, tumor relapse in gastric cancer. These findings are consistent with those of Yan *et al* and Calderaro *et al*, who have shown upregulation of ALDH-3A1 correlated with tumorigenesis in hepatocellular carcinoma and protate cancer, respectively (46,55). It is necessary to clarify the mechanisms underlying ALDH-3A1 prevalence and gastric cancer development. With the recent discovery that EMT transcription factors, such as TWIST could promote cancer stem-like cells by directly binding to E-box sequences in promoter region of CD24 (56), it would be intriguingly to clarify whether the elevated EMT transcription factors would directly act on the ALDH-3A1 promoter region thereby contributing to the maintainance of $ALDH^{bright}$ gastric cancer stem-like subpopulation.

In conclusion, our discoveries identify ALDH-3A1 as a critical CSC marker with potential clinical prognostic application, and demonstrate a clear association between ALDH-3A1 prevalence and gastric cancer evolution.

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