LAMA4 expression is activated by zinc finger E-box-binding homeobox 1 and independently predicts poor overall survival in gastric cancer

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Abstract. The present study aimed to investigate the association between the expression of ZEB1 and LAMA4 in gastric cancer and the possible underlying mechanisms of this. In addition, the present study also investigated the prognostic value of *LAMA4* in gastric cancer. *LAMA4, MMP2, MMP9* and *ZEB1* expression and their associations were analyzed by data mining in The Cancer Genome Atlas-stomach adenocarcinoma (TCGA-STAD). Overall survival (OS) curves of patients with gastric cancer were generated using data from TCGA and Kaplan-Meier plotting. Gastric cancer HGC-27 and SGC-7901 cell lines were used as *in vitro* cell models to assess the effect of LAMA4 on cell migration and invasion and to study the regulatory effect of ZEB1 on LAMA4 expression. The results of the present study indicated that *LAMA4* upregulation was associated with higher grade tumors. *LAMA4*-knockdown significantly reduced MMP2 expression in gastric cancer cells and impaired the speed of wound healing and the invasive capability of the cancer cells. *ZEB1* was strongly co-expressed with *LAMA4* in TCGA-STAD (Pearson's r=0.85). Induced ZEB1 expression significantly increased *LAMA4* expression at the mRNA and protein level in HGC-27 and SGC-7901 cells. A dual-luciferase assay confirmed that ZEB1 directly binded to the promoter of *LAMA4*. High *LAMA4* expression independently predicted a poor OS (HR, 1.614; 95% CI, 1.155-2.256; P=0.005) in patients with primary gastric cancer. These results indicated that ZEB1 was able to epigenetically activate LAMA4 expression via binding to its promoter in gastric cancer cells. High *LAMA4* expression was an independent indicator of a poor OS in patients with gastric cancer.

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Abbreviations: TCGA, The Cancer Genome Atlas; STAD, stomach adenocarcinoma; OS, overall survival; *LAMA4*, laminin subunit α4; ZEB1, zinc finger E-box-binding homeobox 1; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix

Key words: laminin subunit α4, zinc finger E-box-binding homeobox 1, overall survival, gastric cancer

Introduction

Laminins are a family of extracellular matrix (ECM) glycoproteins, which are the major non-collagenous constituent of basement membranes and serve an important role in cell differentiation, migration and adhesion (1,2). In gastric cancer, certain laminin family members are dysregulated and are associated with malignant phenotypes. For example, laminin γ2 upregulation may constitute an adaptive stimulus that allows E-cadherin-defective cells to survive and invade, which contributes toward the subsequent cancer progression (3). Co-expression of laminin β3 and γ2 is significantly correlated with the depth of invasion and advanced tumor stage (4). Epigenetic silencing of laminin β3 chain may reduce cancer cell invasion (4).

The a4 subunit [laminin a4 (LAMA4)] is a component of laminin-8 and laminin-9, which is present in tissues of mesenchymal origin, in endothelial basement membranes and in certain epithelial basement membranes (5). Recent studies have reported that aberrant *LAMA4* expression is associated with enhanced cell migration and metastasis of certain types of cancer, including hepatocellular (6) and breast cancer (7), and renal carcinoma (8). However, the effect of *LAMA4* dysregulation on gastric cancer is poorly understood.

Zinc finger E-box-binding homeobox (ZEB) 1 is an E-box binding transcription factor and is one of the key epithelial-mesenchymal transition (EMT)-inducible genes in multiple types of cancer (9-11). Previous studies have demonstrated that ZEB1 is an independent factor for peritoneal dissemination in patients with gastric cancer (12,13). Knockdown of *ZEB1* can significantly reduce Vimentin expression and increase E-cadherin expression in gastric cancer cells (14), and can also decrease the invasive potential of the cancer cells (15,16). As a transcriptional factor, ZEB1 can act as a transcriptional activator (via binding to CtBP co-repressors) and repressor (via binding to chromatin

remodeling ATPase BRG1, histone acetyl-transferase TIP60 and histone deacetylase SIRT1) (17), depending on specific genes and cells (18,19). In gastric cancer, its downstream regulation remains to be fully elucidated.

The present study investigated the prognostic value and functional role of *LAMA4* in gastric cancer and further investigated the association between the expression of ZEB1 and LAMA4.

Materials and methods

Bioinformatic analysis. The clinicopathological data of patients with primary gastric cancer, the mRNA expression of *LAMA4*, *MMP2*, *MMP9* and *ZEB1,* and their associations in The Cancer Genome Atlas-stomach adenocarcinoma (TCGA-STAD) were analyzed using UCSC Xena Browser (http://xena.ucsc.edu/). The genes co-upregulated with *LAMA4* in TCGA-STAD were also identified using the UCSC browser. LAMA4 protein expression in gastric cancer tissues and in normal gastric tissues was reviewed using immunohistochemistry (IHC) images from the Human Protein Atlas (http://www.proteinatlas.org/) (20), via www.proteinatlas.org/ENSG00000112769-LAMA4/tissue/stomach and http://www.proteinatlas.org/ENSG00000112769-LAMA4/pathology/tissue/stomach+cancer.

The association between *LAMA4* expression and overall survival (OS) of patients with gastric cancer was examined using data in TCGA-STAD and by data mining in Kaplan-Meier plotter (http://kmplot.com/analysis/), an online database containing gene expression data and survival information of 1,065 patients with gastric cancer (21). The patients were divided into two groups by setting the best performing threshold of *LAMA4* expression as the cut-off. The hazard ratio (HR), 95% confidence intervals (CI) and log-rank P-values were calculated. The number-at-risk was indicated below the survival curves.

Cell culture. Human gastric cancer HGC-27 and SGC-7901 cell lines were obtained from the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences (Beijing, China). The cells were cultured with Dulbecco's modified Eagle's medium (DMEM)/high glucose (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin G and 100 μg/ml streptomycin at 37˚C in a humidified 5% CO_2 incubator.

Lentiviral *LAMA4* shRNA particles (SHCLNV-NM_002290) (pLOK.1-CMV-tGFP, with the sequence for shLAMA4-1, 5'-CCGGCGTCTATAATTTGGGAACTAACTCGAGTTAGTTCCCAAATTATAGACGTTTTTG-3' and shLAMA4-2, 5'-CCGGGAACACCACTGACCGAATTTACTCGAGTAAATTCGGTCAGTGGTGTTCTTTTTG-3') and the corresponding negative control (empty pLOK.1-CMV-tGFP) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). ZEB1 lentiviral particles and the corresponding negative controls were purchased from GeneCopoeia (Rockville, MD, USA). The total transducing units needed (TU) for infection was calculated by (total number of cells per well x3). The cancer cells were infected with the lentiviral particles in the presence of Polybrene (8 μg/ml; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocols and were subjected to analysis 48 h later.

Western blot analysis. Conventional western blotting was performed to detect protein band signals. Cells were lysed using a radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) for protein extraction. Protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China). The proteins (25 μg protein/lane) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk for 1 h at room temperature and then incubated with primary antibodies overnight at 4˚C. The primary antibodies used were as follows: Anti-LAMA4 (1:1,000; cat. no. ab209675; Abcam, Cambridge, UK), anti-MMP2 (1:1,000; cat. no. ab37150; Abcam), anti-MMP9 (1:1,000; cat. no. ab38898; Abcam), anti-ZEB1 (1:2,000; cat. no. ab180905; Abcam) and anti-β-actin (1:2,000; cat. no. ab3280; Abcam). Following incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG H&L (1:5,000; cat. no. ab205719; Abcam) or HRP-conjugated goat anti-rabbit IgG H&L (1:10,000; cat. no. ab205718; Abcam) secondary antibody for 1 h in TBST with 5% skimmed milk at room temperature, protein band signals were developed using the enhanced chemiluminescence Plus kit (Amersham, Piscataway, NJ, USA). Band densitometry was performed using ImageJ software (v2.1.4.6; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cell samples using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and were used as the template for reverse transcription with the ProtoScript First Strand cDNA Synthesis kit (New England Biolabs, Ipswich, MA, USA). In brief, RNA was denatured for 5 min at 70˚C. Next, cDNA synthesis reaction was conducted at 42˚C for 1 h. Finally, the enzyme was inactivated at 80˚C for 5 min. Subsequently, qPCR was performed to detect the expression of *LAMA4* mRNA using the SYBR® Select Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences used were as follows: *LAMA4* forward, 5'-GGAAAATAAGCGAGGCACCG-3' and reverse, 5'-AGCCACAGAGGCAGAACCGA-3'; and *GAPDH* forward, 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse, 5'-ACCACCCTGTTGCTGTAGCCAA-3'). The relative expression of *LAMA4* mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (22).

Wound healing assay. In brief, HGC-27 and SGC-7901 cells were cultured in 6-well plates and were infected with lentiviral *LAMA4* shRNA particles or negative controls. A total of 24 h later, confluent cell monolayers were manually wounded by scraping the cells with a 200 μl pipette tip. Wound images were taken at 0 and 24 h after the scratch under an inverted microscope (IX73; Olympus Corporation, Tokyo, Japan), at a magnification of x10. The wound areas were measured using ImageJ software (v2.1.4.7; n=3).

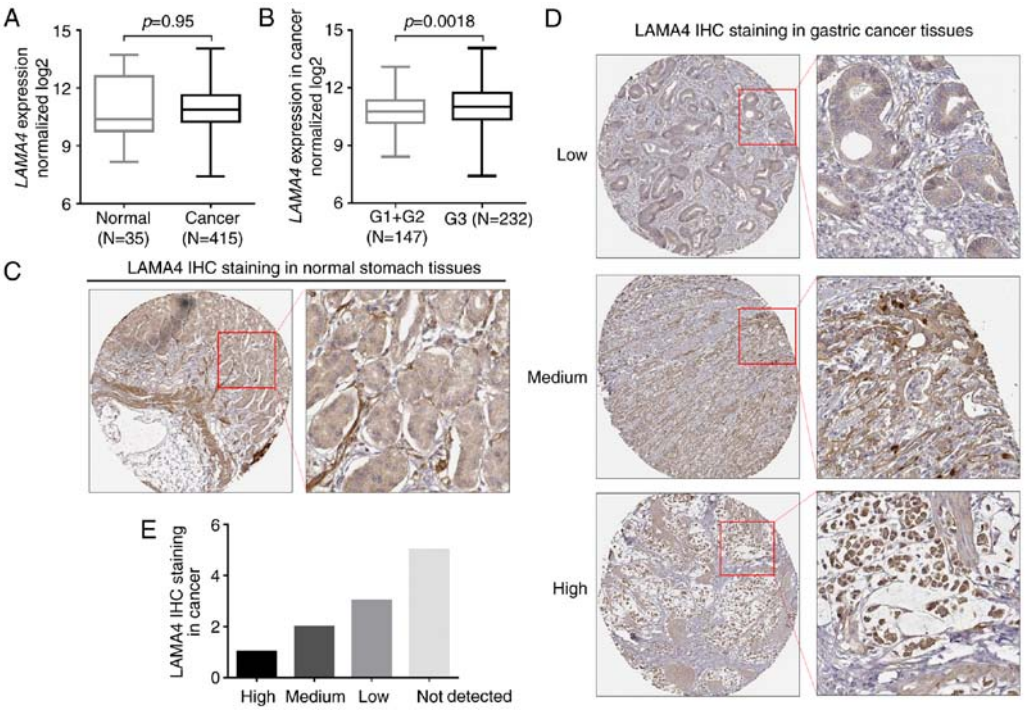

Figure 1. *LAMA4* upregulation is associated with higher tumor grades in gastric cancer. *LAMA4* RNA expression between (A) normal and cancerous stomach tissues, and between (B) G3 (n=232) and G1/G2 (n=147) tumors. (C) Representative image of LAMA4 staining in normal stomach tissues. (D) Representative images and (E) staining summary of LAMA4 in gastric cancer tissues. Images were obtained from the Human Protein Atlas (www.proteinatlas.org/ENSG00000112769-LAMA4/tissue/stomach and http://www.proteinatlas.org/ENSG00000112769-LAMA4/pathology/tissue/stomach+cancer). LAMA4, laminin subunit α4; G, grade; IHC, immunohistochemical.

Transwell assay. A Transwell assay was conducted using a Matrigel invasion chamber (BD Biosciences, San Jose, CA, USA) in a 24-well cell culture plate according to the manufacturer's protocols. Briefly, $3x10^4$ HGC-27 and SGC-7901 cells infected with *LAMA4* shRNA particles or the negative controls were seeded into the upper chamber inserts containing an 8-μm pore size membrane with a thin layer Matrigel matrix, with 500 μl serum-free DMEM. The lower chamber of the well was filled with 700 μl DMEM with 20% FBS. A total of 48 h later, cells that had invaded the lower surface of the membrane were fixed with 70% methanol at room temperature for 10 min while the non-invading cells on the upper surface were removed. The invaded cells were stained with 0.1% crystal violet for 30 min at room temperature, and the number was then determined for 3 independent fields under an inverted microscope (IX73; Olympus Corporation), at a magnification of x100.

Dual-luciferase reporter assay. The promoter sequence of *LAMA4* was obtained from GeneCopoeia (>HPRM34295 NM_001105206). The possible ZEB1 binding sites in the *LAMA4* promoter region were predicted using the JASPAR database (http://jaspar.genereg.net/). *LAMA4* promoter fragments (-1,351 to +219 and -700 to +219) were PCR amplified from the promoter clone (>HPRM34295). The fragments were then inserted into the sites between *Xho*I-*Hin*dIII of pGL3-basic plasmids (Promega Corporation, Madison, WI, USA). 293 cells cultured in 12-well plates were initially infected with lentiviral ZEB1 expression particles or the empty control. A total of 24 h later, the cells were co-transfected with 1.5 μg luciferase construct plasmids (Promega Corporation) or the empty reporter vector DNA and 0.05 μg phRL-TK (Promega Corporation) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 24 h after transfection, the cells were lysed. The luciferase activity of the lysate was measured using the dual-luciferase reporter assay system with a luminometer and was normalized to that of *Renilla* luciferase activity (Promega Corporation, Madison, WI, USA).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). All assays were performed in triplicate and data are reported as the mean ± standard deviation. The group difference was examined by two-tailed Student's t-tests or one-way analysis of variance with Student-Newman-Keuls test as a post hoc test. The association between *LAMA4* RNA expression and the clinicopathological features was assessed using χ^2 tests. Receiver operating characteristic (ROC) curves for mortality were constructed and the optimal cut-off value of *LAMA4* expression was determined based on the Youden index. Log-rank tests were performed to assess the difference between the survival curves. Prognostic values were analyzed by univariate and multivariate Cox regression models. $P<0.05$ was considered to indicate a statistically significant difference.

Results

LAMA4 upregulation is associated with higher grade tumors in gastric cancer. Using RNA-seq data in TCGA-STAD, it was revealed that *LAMA4* RNA expression was not altered in gastric cancer tissues compared with normal stomach tissues (Fig. 1A). However, in the cancer cases, the grade 3 tumors had significantly higher *LAMA4* expression than the

Table I. The association between *LAMA4* expression and the clinicopathological parameters of patients with primary gastric cancer in TCGA-STAD.

	LAMA4 expression			
Parameter	High (n=204)	Low (n=184)	χ^2	P-value
Age, mean ± SD	65.30±10.68	65.30±10.62		1.00
Sex				
Female	67	69	0.92	0.34
Male	137	115		
Histological grade				
G1/G2	65	82	6.62	0.01
G3	134	98		
GX	5	4		
Nodal status				
N0	56	60	0.99	0.32
N+	141	121		
Null	7	3		
Metastasis status				
M0	181	166	0.14	0.71
M1	14	11		
MX	9	7		
Clinical stage				
I/II	81	91	3.01	0.080
III/IV	114	89		
Discrepancy + Null	9	4		
Status				
Alive	106	125	10.25	0.0014
Deceased	98	59		

GX, grade could not be assessed; MX, the presence of distant metastasis could not be assessed; null, no data.

grade 1/2 tumors (P=0.0018; Fig. 1B). By reviewing LAMA4 IHC images in Human Protein Atlas, it was revealed that the LAMA4 staining was usually low in the glandular cells in normal tissues (Fig. 1C). However, the intensity of LAMA4 staining varied significantly in different gastric cancer cases (Fig. 1D). Among 11 gastric cancer tissues, 3 cases had moderate/high LAMA4 staining, while 3 cases had low LAMA4 staining (Fig. 1D).

Knockdown of LAMA4 impaired the migration and invasion of gastric cancer cells. One previous study reported that LAMA4 could promote trophoblast cell invasion and migration via upregulating MMP2 and MMP9, two enzymes facilitating invasion by degrading the ECM (5). By data mining in TCGA-STAD, we the co-expression trend between *LAMA4* and *MMP2* or *MMP9* was characterized (Fig. 2A). Heat-map and subsequent regression analysis demonstrated that *LAMA4* was significantly co-upregulated with *MMP2* (Pearson's r=0.70), but not with MMP9 (Pearson's r=0.18) among the 415 patients with gastric cancer (Fig. 2A). To investigate the functional role of LAMA4 in gastric cancer, HGC-27 and SGC-7901 cells were infected with *LAMA4* shRNA for knockdown (Fig. 2B). In these two cell lines, *LAMA4*-knockdown significantly reduced MMP2 expression, but had little influence on MMP9 expression (Fig. 2C). Wound healing and Transwell assays demonstrated that *LAMA4* inhibition impaired the speed of wound healing (Fig. 2D-E) and reduced the invasive capability of the cancer cells (Fig. 2F-G).

ZEB1 directly increases LAMA4 expression via binding to its promoter. By screening the genes co-upregulated with *LAMA4* in TCGA-STAD, it was revealed that *ZEB1* was correlated with *LAMA4* in gastric cancer (Pearson's r=0.85; Fig. 3A). In fact, *ZEB1* upregulation has well-characterized oncogenic effects on gastric cancer (13,23). By using the UCSC Xena browser (Fig. 3A and B) and the cBioPortal for Cancer Genomics (Fig. 3C), two online tools to analyze data in TCGA-STAD, a strong correlation between the expression of *ZEB1* and that of *LAMA4* was confirmed (Fig. 3A-C). To further investigate the effect of ZEB1 on LAMA4 expression, HGC-27 and SGC-7901 cells were infected with lentiviral *LAMA4* expression particles for overexpression (Fig. 3D). Enforced ZEB1 expression significantly elevated *LAMA4* expression at the mRNA and protein levels (Fig. 3D and E). By promoter scanning, two possible and close ZEB1 binding sites were identified in the promoter of *LAMA4* (Fig. 3F). pGL3-basic-based luciferase reporter plasmids carrying the intact *LAMA4* promoter sequence or truncated sequence

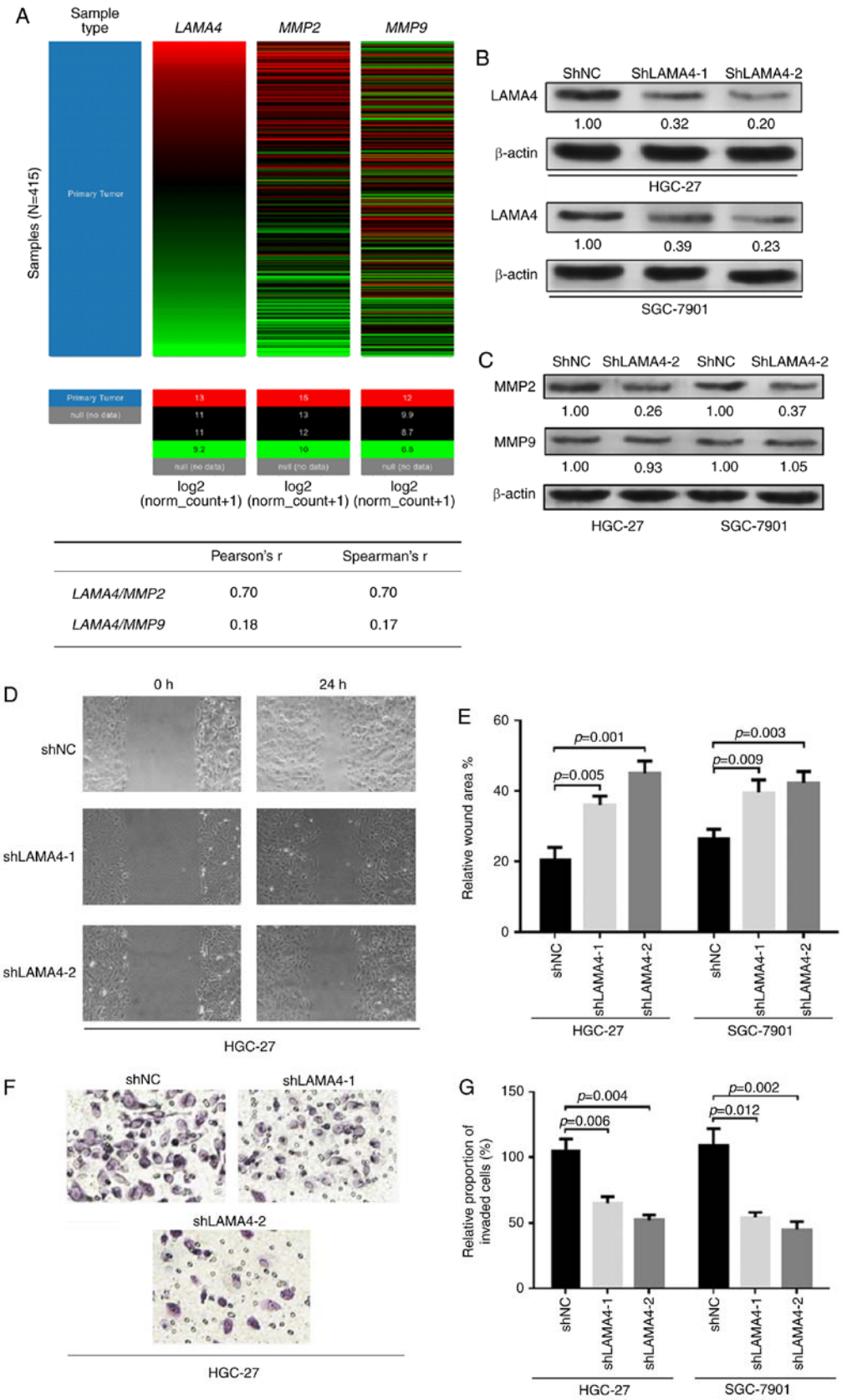

Figure 2. Knockdown of *LAMA4* impaired the migration and invasion of gastric cancer cells. (A) Heat-map of *LAMA4*, *MMP2* and *MMP9* expression in TCGA-STAD (top) and the results of regression analysis (bottom) between *LAMA4* and *MMP2* or *MMP9*. (B) Western blot analysis of LAMA4 protein expression in HGC-27 and SGC-7901 cells 36 h after infection with *LAMA4* lentiviral shRNA. (C) Western blot analysis of MMP2 and MMP9 expression in HGC-27 and SGC-7901 cells 48 h after infection with *LAMA4* lentiviral shRNA. (D) Representative images and (E) quantitation results of wound healing assay. (F) Representative images and (G) quantitation results of Transwell assay, which were conducted 48 h after infection of HGC-27 and SGC-7901 cells with *LAMA4* lentiviral shRNA. (D and E) The relative wound areas at 24 h compared with 0 h after scratching were calculated to reflect the speed of wound healing. (F and G) The relative proportion of invaded cells in *LAMA4* shRNA groups compared with shNC groups were calculated to reflect the capability of cell invasion. LAMA4, laminin subunit α4; MMP, matrix metalloproteinase; TCGA-STAD, The Cancer Genome Atlas-stomach adenocarcinoma; shRNA, short hairpin RNA; NC, negative control.

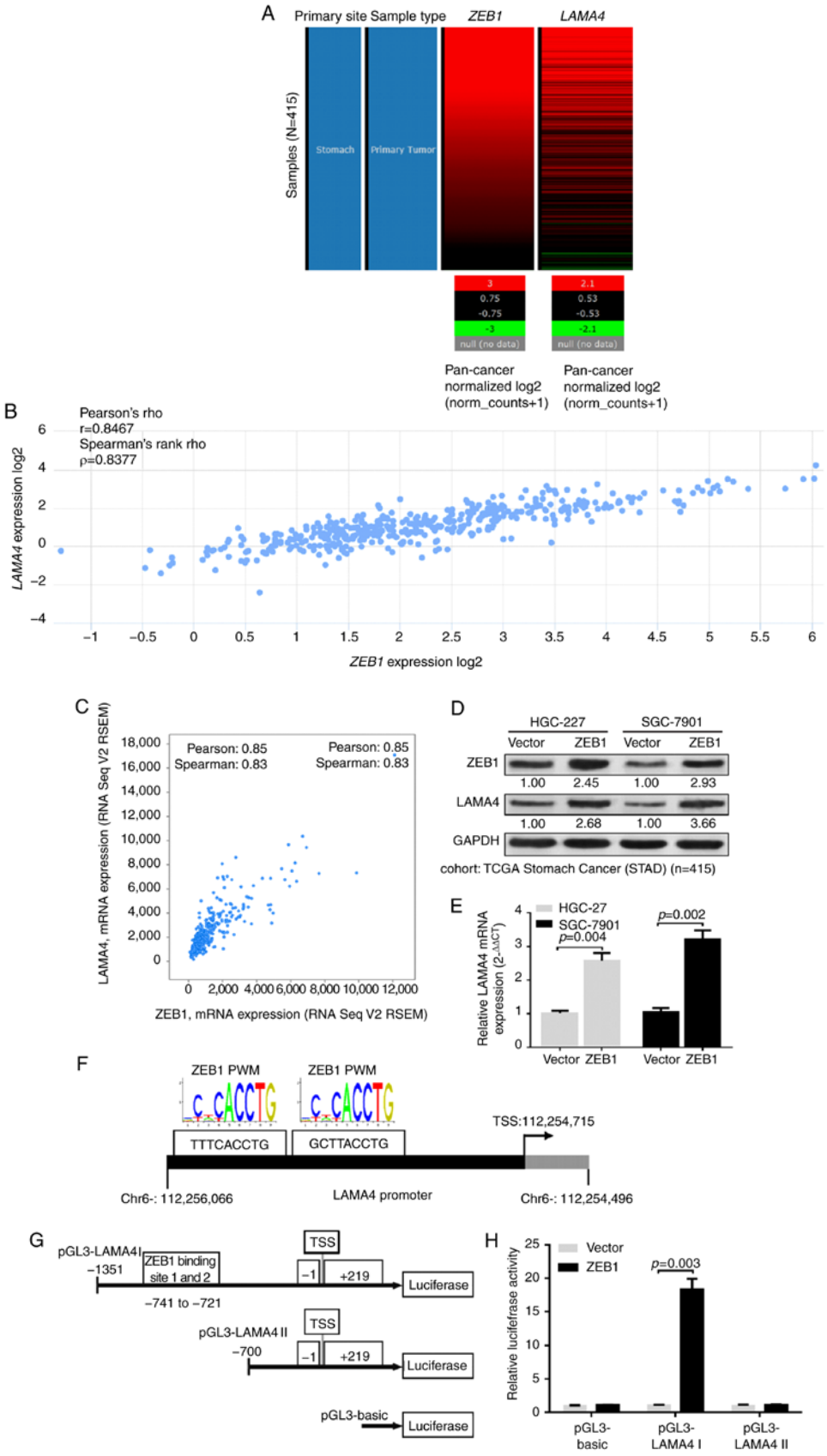

Figure 3. ZEB1 directly increases *LAMA4* expression via binding to its promoter. (A) Heat-map and (B) regression analysis of the correlation between *ZEB1* and *LAMA4*. Data analysis was performed by using the UCSC Xena browser. (C) Regression analysis of the correlation between *ZEB1* and *LAMA4*. Data analysis was performed using the cBioPortal for Cancer Genomics. (D) Western blot analysis of ZEB1 and LAMA4 expression in HGC-27 and SGC-7901 cells 48 h after infection of ZEB1 lentiviral expression particles or empty controls. (E) qRT-PCR analysis of LAMA4 mRNA expression in HGC-27 and SGC-7901 cells 48 h after infection of ZEB1 lentiviral expression particles or empty controls. (F) Predicted ZEB1 binding sites in the *LAMA4* promoter. (G) Design of reconstructed pGL3-basic plasmids carrying intact or truncated LAMA4 promoter fragments. (H) The luciferase reporter constructs carrying intact or truncated *LAMA4* promoter sequences were introduced into 293 cells pre-infected with lentiviral ZEB1 expression particles or the empty control. Luciferase activity was measured 24 h post-transfection. ZEB1, zinc finger E-box-binding homeobox 1; LAMA4, laminin subunit α4; TCGA, The Cancer Genome Atlas.

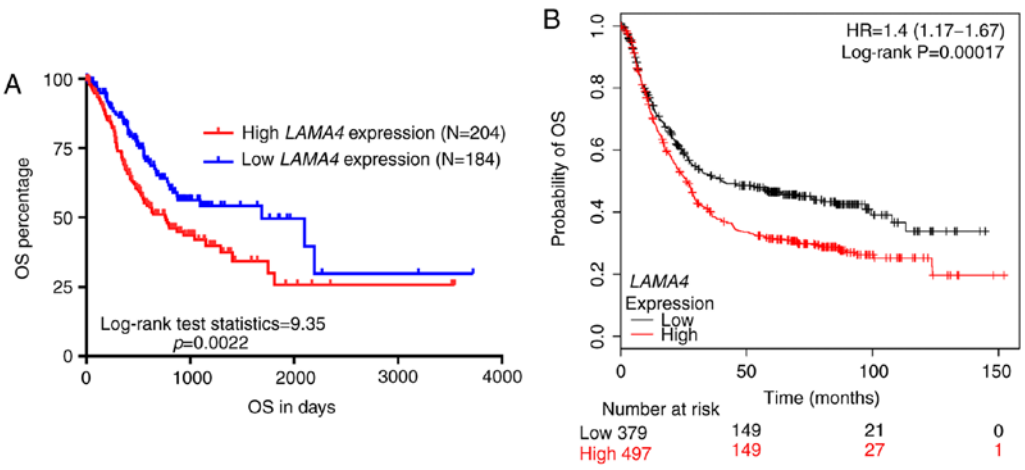

Figure 4. *LAMA4* upregulation is associated with unfavorable OS in patients with gastric cancer. Kaplan-Meier curves of OS in patients with gastric cancer grouped by high and low *LAMA4* expression. Survival curves were generated by (A) using data from TCGA-STAD or (B) by data mining in Kaplan-Meier plotter.

Table II. Univariate and multivariate analyses of overall survival in patients with primary gastric cancer in TCGA-STAD.

	Univariate analysis				Multivariate analysis			
Parameter	P-value	HR	95% CI (lower/upper)		P-value	HR	95% CI (lower/upper)	
Age								
>65 vs. ≤65	0.006	1.572	1.139	2.171	0.002	2.001	1.421	2.816
Sex								
Female vs. male	0.296	0.835	0.596	1.171				
Grade								
G3/G4 vs. G1/G2	0.022	1.479	1.057	2.069	0.039	1.463	1.020	2.098
Nodal status								
N1+ vs. N0	0.001	2.042	1.362	3.061	0.136	1.526	0.876	2.661
Metastasis status								
M1 vs. M0	0.002	2.334	1.367	3.986	0.002	2.429	1.371	4.305
Clinical stage								
III/IV vs. I/II	<0.001	2.063	1.460	2.916	0.173	1.398	0.863	2.265
LAMA4 expression								
High vs. low	0.002	1.664	1.205	2.300	0.005	1.614	1.155	2.256

were generated (Fig. 3G). A luciferase assay revealed that ZEB1 overexpression significantly increased the luciferase activity of the reporter with the intact *LAMA4* promoter sequence (Fig. 3H). By comparison, *ZEB1* overexpression had little influence on the luciferase activity of the reporter with the truncated *LAMA4* promoter sequence (Fig. 3H).

High LAMA4 expression independently predicts a poor OS in patients with primary gastric cancer. In order to investigate the prognostic value of *LAMA4* in gastric cancer, the association between *LAMA4* expression and OS was further assessed based on data in TCGA-STAD and by data mining in Kaplan-Meier plotter. The associations between *LAMA4* expression and the clinicopathological parameters in patients with primary gastric cancer in TCGA were summarized in Table I. The high *LAMA4* expression group had significantly higher ratios of grade 3 (G3) tumors (134/199, 67.3%) and mortality (98/204, 48.0%) than the low *LAMA4* expression group (G3, 98/180, 54.4%; mortality, 59/184, 32.1%; Table I). Kaplan-Meier curves demonstrated that the high *LAMA4* expression group (n=204) had significantly poorer OS rates than the low *LAMA4* expression group (n=184; P=0.0022; Fig. 4A). Data mining in Kaplan-Meier plotter also confirmed this association (HR, 1.4; 95% CI, 1.17-1.67; P<0.001; Fig. 4B). In univariate analysis, it was revealed that high age (>65), high grade (G3/G4), nodal invasion, metastasis, advanced disease stage (III/IV) and high *LAMA4* expression were associated with significantly shorter OS times (Table II). Multivariate

analysis revealed that the high *LAMA4* expression could independently predict a poor OS (HR, 1.614; 95% CI, 1.155-2.256; P=0.005; Table II).

Discussion

In the present study, the results of bioinformatic analysis indicated that *LAMA4* upregulation was associated with higher grades of gastric cancer. *LAMA4* upregulation is associated with enhanced invasion and metastasis of cancer cells. In hepatocellular carcinoma, LAMA4 has specific *in vivo* distribution in the tumor basement membrane and its upregulation is correlated with tumor invasion and metastasis (6). In renal cell carcinoma, *LAMA4* is upregulated in locally advanced tumors and in primary tumor and secondary metastases (8). *LAMA4* upregulation may also predict poor survival in patients with renal cell carcinoma (8). One recent study reported that LAMA4 could promote trophoblast cell invasion and migration via upregulating MMP2 and MMP9 (5). MMP2 and MMP9 are two critical enzymes degrading ECM, thereby supporting cancer cell migration and invasion (24,25). In fact, trophoblast research over the past decades revealed that placental cells have high levels of similarities in proliferative, migratory and invasive properties to those of cancer cells (26). As LAMA4 dysregulation may be associated with tumor grade in gastric cancer, the present study investigated its regulative effect on the migration and invasion of gastric cancer cells. In HGC-27 and SGC-7901 cells, *LAMA4*-knockdown significantly reduced MMP2 expression, but had little influence on MMP9 expression. Functional assays revealed that *LAMA4* inhibition impaired the speed of wound healing and also reduced the invasive capability of the cancer cells.

To investigate the mechanism of LAMA4 dysregulation in gastric cancer, we identified the genes significantly co-expressed with *LAMA4* in TCGA-STAD and observed that *ZEB1* is correlated with *LAMA4* expression. The oncogenic effects of aberrant ZEB1 expression in gastric cancer have been widely reported (13,15,27). As a transcription factor, ZEB1 can modulate the expression of a series of genes in different types of cancer. In breast cancer, ZEB1 can upregulate VEGF expression and promote angiogenesis (28). Additionally, ZEB1 can reduce *NGN3* transcription via forming a ZEB1/DNA methyltransferase (DNMT)3B/histone deacetylase 1 (HDAC1) complex on the *NGN3* promoter (29). In tongue cancer cells, ZEB1 can bind to the CA9 promoter and positively regulate its expression, thereby leading to enhanced chemoresistance (30). In gallbladder cancer cells, ZEB1 can repress T-cadherin expression via binding to the promoter, thereby increasing their invasive capability (31). These results suggested that ZEB1 can be either an epigenetic activator or repressor, depending on specific gene and cancer types. However, the regulative effect of ZEB1 in gastric cancer is not yet fully understood. The present study revealed that ZEB1 can directly increase *LAMA4* expression via binding to its promoter in gastric cancer cells. This finding revealed a novel regulative effect of ZEB1 in gastric cancer.

Based on data mining in two large databases, including TCGA-STAD and Kaplan-Meier plotter, it was revealed that *LAMA4* upregulation is associated with unfavorable OS rates in patients with gastric cancer. Univariate and multivariate analysis demonstrated that the high *LAMA4* expression could independently predict a poor OS rate (HR, 1.614; 95% CI, 1.155-2.256; P=0.005), suggesting that *LAMA4* expression may be a valuable biomarker in gastric cancer.

Based on the aforementioned results, we hypothesized that ZEB1 could epigenetically activate *LAMA4* expression via binding to its promoter in gastric cancer cells, while high *LAMA4* expression was an independent indicator for a poor OS in patients with gastric cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XW and QH performed cellular studies and conducted data analysis and interpretation. XW and XZ collected and analyzed data from databases. All authors participated in the manuscript preparation and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Cheng YS, Champliaud MF, Burgeson RE, Marinkovich MP and Yurchenco PD: Self-assembly of laminin isoforms. J Biol Chem 272: 31525-31532, 1997.
2. Lugassy C, Torres-Munoz JE, Kleinman HK, Ghanem G, Vernon S and Barnhill RL: Overexpression of malignancy-associated laminins and laminin receptors by angiotropic human melanoma cells in a chick chorioallantoic membrane model. J Cutan Pathol 36: 1237-1243, 2009.
3. Caldeira J, Figueiredo J, Bras-Pereira C, Carneiro P, Moreira AM, Pinto MT, Relvas JB, Carneiro F, Barbosa M, Casares F, *et al*: E-cadherin-defective gastric cancer cells depend on Laminin to survive and invade. Hum Mol Genet 24: 5891-5900, 2015.
4. Ii M, Yamamoto H, Taniguchi H, Adachi Y, Nakazawa M, Ohashi H, Tanuma T, Sukawa Y, Suzuki H, Sasaki S, *et al*: Co-expression of laminin beta3 and gamma2 chains and epigenetic inactivation of laminin alpha3 chain in gastric cancer. Int J Oncol 39: 593-599, 2011.
5. Shan N, Zhang X, Xiao X, Zhang H, Tong C, Luo X, Chen Y, Liu X, Yin N, Deng Q and Qi H: Laminin alpha4 (LAMA4) expression promotes trophoblast cell invasion, migration, and angiogenesis, and is lowered in preeclamptic placentas. Placenta 36: 809-820, 2015.

6. Huang X, Ji G, Wu Y, Wan B and Yu L: LAMA4, highly expressed in human hepatocellular carcinoma from Chinese patients, is a novel marker of tumor invasion and metastasis. J Cancer Res Clin Oncol 134: 705-714, 2008.
7. Ross JB, Huh D, Noble LB and Tavazoie SF: Identification of molecular determinants of primary and metastatic tumour re-initiation in breast cancer. Nat Cell Biol 17: 651-664, 2015.
8. Wragg JW, Finnity JP, Anderson JA, Ferguson HJ, Porfiri E, Bhatt RI, Murray PG, Heath VL and Bicknell R: MCAM and LAMA4 are highly enriched in tumor blood vessels of fenal cell carcinoma and predict patient outcome. Cancer Res 76: 2314-2326, 2016.
9. Spaderna S, Schmalhofer O, Wahlbuhl M, Dimmler A, Bauer K, Sultan A, Hlubek F, Jung A, Strand D and Eger A: The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. Cancer Res 68: 537-544, 2008.
10. Wellner U, Schubert J, Burk UC, Schmalhofer O, Zhu F, Sonntag A, Waldvogel B, Vannier C, Darling D, zur Hausen A, *et al*: The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat Cell Biol 11: 1487-1495, 2009.
11. Guo E, Wang Z and Wang S: MiR-200c and miR-141 inhibit ZEB1 synergistically and suppress glioma cell growth and migration. Eur Rev Med Pharmacol Sci 20: 3385-3391, 2016.
12. Okugawa Y, Toiyama Y, Tanaka K, Matsusita K, Fujikawa H, Saigusa S, Ohi M, Inoue Y, Mohri Y, Uchida K and Kusunoki M: Clinical significance of Zinc finger E-box Binding homeobox 1 (ZEB1) in human gastric cancer. J Surg Oncol 106: 280-285, 2012.
13. Jia B, Liu H, Kong Q and Li B: Overexpression of ZEB1 associated with metastasis and invasion in patients with gastric carcinoma. Mol Cell Biochem 366: 223-229, 2012.
14. Hur K, Toiyama Y, Takahashi M, Balaguer F, Nagasaka T, Koike J, Hemmi H, Koi M, Boland CR and Goel A: MicroRNA-200c modulates epithelial-to-mesenchymal transition (EMT) in human colorectal cancer metastasis. Gut 62: 1315-1326, 2013.
15. Zhou X, Wang Y, Shan B, Han J, Zhu H, Lv Y, Fan X, Sang M, Liu XD and Liu W: The downregulation of miR-200c/141 promotes ZEB1/2 expression and gastric cancer progression. Med Oncol 32: 428, 2015.
16. Yanaka Y, Muramatsu T, Uetake H, Kozaki K and Inazawa J: *miR-544a* induces epithelial-mesenchymal transition through the activation of WNT signaling pathway in gastric cancer. Carcinogenesis 36: 1363-1371, 2015.
17. Sanchez-Tillo E, Liu Y, de Barrios O, Siles L, Fanlo L, Cuatrecasas M, Darling DS, Dean DC, Castells A and Postigo A: EMT-activating transcription factors in cancer: Beyond EMT and tumor invasiveness. Cell Mol Life Sci 69: 3429-3456, 2012.
18. Postigo AA and Dean DC: ZEB represses transcription through interaction with the corepressor CtBP. Proc Natl Acad Sci USA 96: 6683-6688, 1999.
19. Larsen JE, Nathan V, Osborne JK, Farrow RK, Deb D, Sullivan JP, Dospoy PD, Augustyn A, Hight SK and Sato M: ZEB1 drives epithelial-to-mesenchymal transition in lung cancer. J Clin Invest 126: 3219-3235, 2016.
20. Lindskog C: The potential clinical impact of the tissue-based map of the human proteome. Expert Rev Proteomics 12: 213-215, 2015.
21. Szász AM, Lánczky A, Nagy Á Förster S, Hark K, Green JE, Boussioutas A, Busuttil R, Szabó A and Győrffy B: Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients. Oncotarget 7: 49322-49333, 2016.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408, 2001.
23. Yabusaki N, Yamada S, Murai T, Kanda M, Kobayashi D, Tanaka C, Fujii T, Nakayama G, Sugimoto H, Koike M, *et al*: Clinical significance of zinc-finger E-box binding homeobox 1 mRNA levels in peritoneal washing for gastric cancer. Mol Clin Oncol 3: 435-441, 2015.
24. Sato H and Seiki M: Membrane-type matrix metalloproteinases (MT-MMPs) in tumor metastasis. J Biochem 119: 209-215, 1996.
25. Yasui W, Oue N, Aung PP, Matsumura S, Shutoh M and Nakayama H: Molecular-pathological prognostic factors of gastric cancer: A review. Gastric Cancer 8: 86-94, 2005.
26. Ferretti C, Bruni L, Dangles-Marie V, Pecking AP and Bellet D: Molecular circuits shared by placental and cancer cells, and their implications in the proliferative, invasive and migratory capacities of trophoblasts. Hum Reprod Update 13: 121-141, 2007.
27. Miao L, Xiong X, Lin Y, Cheng Y, Lu J, Zhang J and Cheng N: Down-regulation of FoxM1 leads to the inhibition of the epithelial-mesenchymal transition in gastric cancer cells. Cancer Genet 207: 75-82, 2014.
28. Liu L, Tong Q, Liu S, Cui J, Zhang Q, Sun W and Yang S: ZEB1 upregulates VEGF expression and stimulates angiogenesis in breast cancer. PLoS One 11: e0148774, 2016.
29. Zhou C, Jiang H, Zhang Z, Zhang G, Wang H, Zhang Q, Sun P, Xiang R and Yang S: ZEB1 confers stem cell-like properties in breast cancer by targeting neurogenin-3. Oncotarget 8: 54388-54401, 2017.
30. Zheng G, Peng C, Jia X, Gu Y, Zhang Z, Deng Y, Wang C, Li N, Yin J, Liu X, *et al*: ZEB1 transcriptionally regulated carbonic anhydrase 9 mediates the chemoresistance of tongue cancer via maintaining intracellular pH. Mol Cancer 14: 84, 2015.
31. Adachi Y, Takeuchi T, Nagayama T, Ohtsuki Y and Furihata M: Zeb1-mediated T-cadherin repression increases the invasive potential of gallbladder cancer. FEBS Lett 583: 430-436, 2009.