

# E-cadherin is downregulated by microenvironmental changes in pancreatic cancer and induces EMT

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**Abstract.** The aim of the present study was to research the effect of microenvironmental change on epithelial-mesenchymal transition (EMT) in pancreatic cancer cells and to determine the correlation between E-cadherin expression and the prognosis of pancreatic cancer patients. We established hypoxic, serum-deficient and TGF- $\beta$ -induced microenvironment models of pancreatic cancer cells and studied the changes in the mRNA and protein expression of EMT-related molecules, E-cadherin and vimentin, using western blot analysis and real-time PCR. Furthermore, immunohistochemistry was used to investigate E-cadherin expression in pancreatic cancer tissues, and survival analysis and COX regression analysis were conducted. In pancreatic cancer cells under hypoxic, serum-starved and TGF- $\beta$ -induced microenvironments, E-cadherin protein and mRNA levels were significantly decreased ( $P < 0.05$ ), while vimentin protein and mRNA expression levels were significantly increased ( $P < 0.05$ ). The results of immunohistochemistry showed that the protein level of E-cadherin in pancreatic cancer tissues was positively correlated with overall survival ( $P < 0.01$ ). The results of Cox regression analysis showed that E-cadherin was an independent prognostic factor in pancreatic cancer. In conclusion, E-cadherin expression was significantly decreased by microenvironment changes, and this decrease induced EMT in pancreatic cancer cells. E-cadherin is an independent prognostic marker in pancreatic cancer patients.

## Introduction

The pathogenesis of pancreatic cancer is opaque, its progress is rapid, its prognosis and therapeutic effects are poor,

its incidence is almost equal to its mortality rate, and the vast majority of patients die due to metastases. Pancreatic cancer still has the fourth highest death rate worldwide (1,2). The early metastasis of pancreatic cancer is an extremely complex, dynamic process with multi-factor participation and multi-stage development. The exact molecular mechanism responsible is still not clear. Many studies have shown that many factors, including epithelial-mesenchymal transition (EMT) (3,4), the tumor microenvironment (5,6), inflammatory factors (7,8), stress response (9), and circulating tumor cells (CTCs) (10) play vital roles in the process of pancreatic cancer metastasis.

EMT is the 'engine' that leads to the invasion and metastasis of pancreatic cancer (11). The process is accompanied by changes in the expression of multiple genes. The most significant changes are in the epithelial marker E-cadherin and mesenchymal marker vimentin. Microenvironmental changes in pancreatic cancer are the primary causes of EMT. In the present study, we induced changes in the microenvironment of pancreatic cancer cells *in vitro*, such as making it anaerobic, making it serum-deficient, and adding TGF- $\beta$ , and then observed the changes of the protein and mRNA expression levels of EMT markers E-cadherin and vimentin. Furthermore, we conducted a survival analysis and Cox regression analysis to investigate the correlation between the expression level of E-cadherin and overall survival.

## Materials and methods

**Cell lines.** Eight human pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-1, Colo-357, MIA PaCa-2, Panc-1, SU86.86 and T3M4, were kindly donated by Professor Helmut Freiss from the Technical University Munich of Germany. All the cell lines were individually cultured in a humidified incubator with 5% CO<sub>2</sub> at 37°C in either RPMI-1640 medium or Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA, USA).

**Clinical samples.** All 52 pancreatic tissues (Table I), including 25 male cases and 27 female cases, age distribution from 41.4 to 81.1 years (65.59 $\pm$ 1.21 years), were obtained from the Department of General Surgery, Peking Union Medical College Hospital (PUMCH), China. The date range of recruitment

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was from January 2012 to December 2017. The experimental protocol was approved by the Peking Union Medical College Hospital Ethics Committee. Written informed consent was obtained from the patients. Tissue sampling and processing were performed as previously described (12).

**RNA extraction and quantitative real-time PCR (qPCR).** Total RNA was extracted from cells and tissues using Invitrogen™ TRIzol reagent (Thermo Fisher Scientific, Inc. Waltham, MA, USA), according to the manufacturer's instructions. cDNA was synthesized using Invitrogen™ M-MLV reverse transcriptase (Thermo Fisher Scientific, Inc.) from 5 µg total RNA. Quantitative RT-PCR was performed on the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Foster City, CA, USA) using KAPA PROBE FAST qPCR kits (Kapa Biosystems, Inc., Wilmington, MA, USA) and TaqMan probes (Thermo Fisher Scientific, Inc.) with the following cycling conditions: 95°C for 10 min (initial denature) and then 40 cycles of 95°C for 15 sec, 60°C for 60 sec. The expression of genes was calculated using the  $2^{-\Delta\Delta C_q}$  method (13). The sequences of E-cadherin primers used were as follows: 5'-CTGAGAACG AGGCTAACG-3' and 5'-TTCACATCCAGCACATCC-3'; the sequences of vimentin primers used were as follows: 5'-CCA GGCAAAGCAGGAGTC-3' and 5'-CGAAGGTGACGAGCC ATT-3'. Reference gene was GAPDH, the sequences of GAPDH primers used were as follows: 5'-TCAACGACCACTTTGTCA AGCTCA-3' and 5'-TCATTCTGGGGACCTGGTGGTCG-3'.

**Immunoblot analysis.** For immunoblot analysis, cells were grown in 6-well plates and processed as previously described (1). The primary antibodies were applied overnight at 4°C, and the secondary antibodies were added and incubated for 1 h at room temperature. The E-cadherin antibody (cat. no. sc-21791), HIF-1α antibody (cat. no. sc-71247) and vimentin antibody (cat. no. sc-66002) (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were diluted at 1:1,000. The GAPDH antibody was diluted at 1:5,000 and used to verify equal loading.

**Serum starvation, hypoxia and TGF-β induction.** For serum starvation, Colo-357 and BxPC-3 cells were seeded in 6-well plates; after the cells reached 70% confluency, the medium was changed to either 0.5 or 10% FCS containing medium that was kept on the cells for the indicated periods of time. For hypoxia, sister clones of Colo-357 cells were incubated under normoxic and hypoxic conditions (89.25% N<sub>2</sub> + 10% CO<sub>2</sub> + 0.75% O<sub>2</sub>) for 24 and 48 h at 37°C in medium supplemented with 10% FCS, as previously described (13,19,21). For TGF-β induction, sister clones of Colo-357 and BxPC-3 cells were incubated with TGF-β (ab50036, terminal concentration 10 ng/ml; Abcam, Cambridge, UK) or not. All experiments were performed in triplicate and repeated three times.

**Immunohistochemical analysis.** Semi-quantitative immunohistochemistry (IHC) was performed to determine E-cadherin expression in 10 normal pancreas and 52 pancreatic cancer samples according to a previously published standard protocol (2). The E-cadherin antibody was diluted at 1:200. Two pathologists who specialized in pancreatic cancer independently rated the staining intensity and percentage of stained

cells for each sample. Briefly, scores were applied to rate staining intensity in the cancer cells (no staining, 0; weak, 1; moderate, 2; strong, 3) and to determine the percentage of stained cells (<5%, 0; 5-25%, 1; >25-50%, 2; >50-75%, 3; >75%, 4). The final intensity score was equal to the staining intensity multiplied by the cell percentage. The staining was stratified accordingly to low levels of expression (scores 1-3) or high levels of expression (score ≥4).

**Statistical analysis.** Statistical analyses were performed using SPSS 23.0 software (IBM Corp., Armonk, NY, USA). Median values were used as cut-off limits for group comparisons. Survival analyses were performed using the Kaplan-Meier method and log-rank tests. Multivariable analysis was performed using a Cox proportional hazards model. The median survival and estimations of hazard ratios were reported with 95% confidence intervals. Comparisons of demographic and clinicopathological data between groups were made using a Chi-square test. The data are presented as the mean ± standard deviation (SD) and were compared using either Student's t test, the Mann-Whitney U test and the one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference post hoc test. Statistical significance was set at a P-value of <0.05. Graph-Pad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA) software was used to create graphs.

## Results

*E-cadherin protein and mRNA are expressed in several of 8 pancreatic cancer cell lines, and the expression was decreased by hypoxia, inversely to vimentin.* We first detected the expression of E-cadherin in 8 pancreatic cancer cell lines with western blotting and real-time PCR. The results showed that E-cadherin mRNA and protein were distinctly detectable in some of these cell lines, with high expression in T3M4, BxPC-3 and Colo-357 cells and low expression in MIA PaCa-2, Panc-1 and SU86.86 cells (Fig. 1A and B). In order to further research this topic using siRNA and overexpression plasmid, the cell lines with relatively high expression, BxPC-3 and Colo-357 were chosen for further functional experiments.

To analyze whether hypoxia decreases the expression of E-cadherin, cancer cells were exposed to a hypoxic environment. Cellular hypoxia was verified by increased HIF-1α protein expression. When compared with the normoxic controls, E-cadherin protein in Colo-357 cells was decreased 1.7- and 2.6-fold (P<0.01) due to hypoxia at 24 and 48 h, respectively (Fig. 1C), and in BxPC-3 cells, E-cadherin protein was decreased 1.2- and 2.1-fold (P<0.01) due to hypoxia at 24 and 48 h, respectively (Fig. 1F). Similarly, in Colo-357 cells, there were 4.7 and 7.8-fold (P<0.01) decreases at the mRNA level due to hypoxia at 24 and 48 h, respectively (Fig. 1D), and in BxPC-3 cells, there were 1.2- and 4.6-fold (P<0.01) decreases at the mRNA level of E-cadherin due to hypoxia at 24 and 48 h, respectively (Fig. 1G). In contrast, in Colo357 cells, vimentin protein was increased 1.9- and 3.1-fold (P<0.01) due to hypoxia at 24 and 48 h, respectively (Fig. 1C), and in BxPC-3 cells, vimentin protein was increased 1.8- and 1.9-fold (P<0.05) due to hypoxia at 24 and 48 h, respectively (Fig. 1F). Similarly, there were 1.3- and 2.1-fold (P<0.01) increases at the

Table I. Association between E-cadherin expression and clinicopathological features in PDAC patients.

Variables	E-cadherin weak expression	E-cadherin strong expression	Mann-Whitney t-test	Chi-square
No. of patients	30	22		
Male vs. female	17/13	8/14		NS
Age (years)	Median=66.4	Median=65.3	NS	
T1 & T2 vs. T3	1/29	3/19		NS
N0 vs. N1	5/25	9/13		NS
G1	1	4		
G2	21	12		
G3	8	6		
G1 vs. G2 & G3	1 vs. 29	4 vs. 18		NS

NS, not significant; PDAC, pancreatic ductal adenocarcinoma.

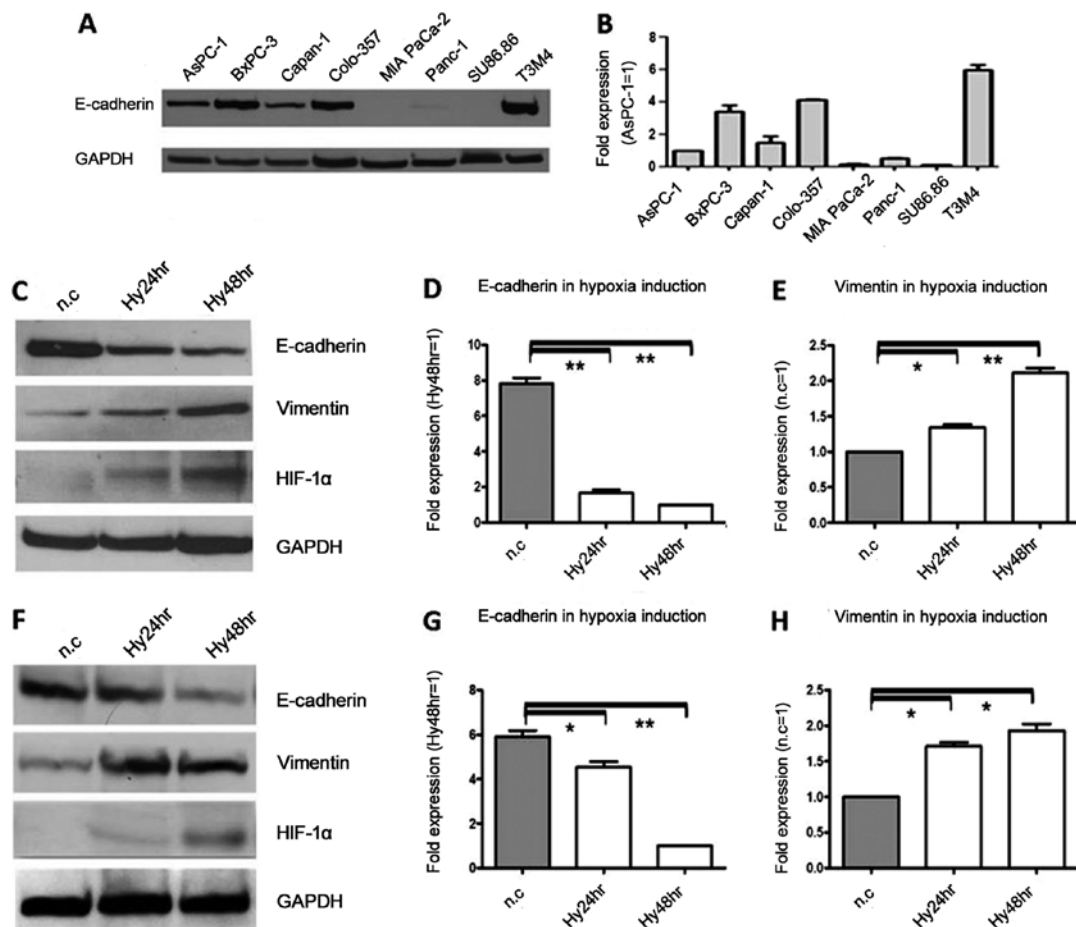


Figure 1. E-cadherin protein and mRNA expression in 8 pancreatic cancer cell lines, and downregulation during hypoxia, in contrast to vimentin. (A) Western blot results showed that E-cadherin protein was highly expressed in T3M4, BxPC-3 and Colo-357 cell lines, whereas E-cadherin protein was expressed at a low level in MIA PaCa-2, Panc-1 and Su86.86 cell lines. GAPDH was used as the loading control. (B) Real-time PCR results showed that E-cadherin mRNA was differentially expressed in eight cell lines, and is presented as a magnitude of relative expression, which was in accordance with the western blot results.  $\beta$ -actin was used as the housekeeping gene. (C) The protein expression levels of E-cadherin and vimentin after exposure to hypoxia for 24 and 48 h were analyzed by immunoblot analysis in Colo-357 cells. E-cadherin protein was decreased 1.7- and 2.6-fold ( $P < 0.01$ ), respectively. In contrast, vimentin protein was increased 1.9- and 3.1-fold ( $P < 0.01$ ), respectively. GAPDH was used as the loading control. (D) The mRNA expression levels of E-cadherin after exposure to hypoxia were analyzed by real-time PCR in Colo-357 cells, and there were 4.7- and 7.8-fold ( $P < 0.01$ ) decreases at 24 and 48 h, respectively.  $\beta$ -actin was used as the housekeeping. (E) The mRNA expression levels of vimentin after exposure to hypoxia were analyzed by real-time PCR in Colo-357 cells, and there were 1.3- and 2.1-fold ( $P < 0.01$ ) increases at 24 and 48 h, respectively.  $\beta$ -actin was used as the housekeeping. (F) The protein expression levels of E-cadherin and vimentin after exposure to hypoxia for 24 and 48 h were analyzed by immunoblot analysis in BxPC-3 cells. E-cadherin protein was decreased 1.2- and 2.1-fold ( $P < 0.01$ ), respectively. In contrast, vimentin protein was increased 1.8- and 1.9-fold ( $P < 0.05$ ), respectively. GAPDH was used as the loading control. (G) The mRNA expression levels of E-cadherin after exposure to hypoxia were analyzed by real-time PCR in BxPC-3 cells, and there were 1.2- and 4.6-fold ( $P < 0.01$ ) decreases at 24 and 48 h, respectively.  $\beta$ -actin was used as the housekeeping. (H) The mRNA expression levels of vimentin after exposure to hypoxia were analyzed by real-time PCR in BxPC-3 cells, and there were 1.7- and 1.9-fold ( $P < 0.05$ ) increases at 24 and 48 h, respectively.  $\beta$ -actin was used as the housekeeping. Results are expressed as the magnitude of relative expression (means  $\pm$  SEM) compared with the control, from three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ . n.c., negative control; Hy24hr, hypoxia for 24 h; Hy48hr, hypoxia for 48 h.

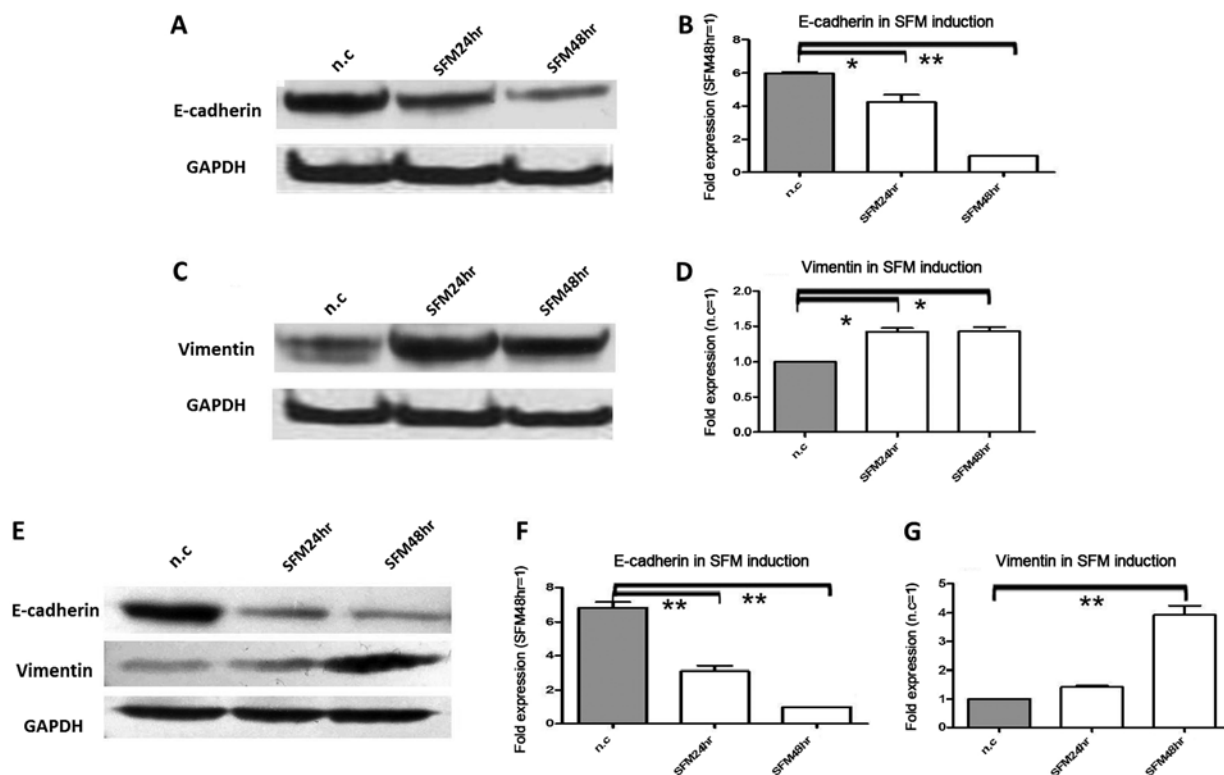


Figure 2. The protein and mRNA expression changes in E-cadherin and vimentin under serum starvation. Pancreatic cancer cells Colo-357 and BxPC-3 were cultured under serum starvation (FCS 0.5%) for 24 and 48 h. (A) E-cadherin protein was decreased 1.5-fold ( $P<0.05$ ) and 3.3-fold ( $P<0.01$ ) at 24 and 48 h in Colo-357 cells, respectively. (B) E-cadherin mRNA was decreased 1.4-fold ( $P<0.05$ ) and 5.9-fold ( $P<0.01$ ) at 24 and 48 h in Colo-357 cells, respectively. (C) Vimentin protein was increased 2.4-fold ( $P<0.01$ ) and 2.8-fold ( $P<0.01$ ) at 24 and 48 h in Colo-357 cells, respectively. (D) Vimentin mRNA was increased 1.4-fold ( $P<0.05$ ) and 1.6-fold ( $P<0.05$ ) at 24 and 48 h in Colo-357 cells, respectively. (E) E-cadherin protein was decreased 1.6-fold ( $P<0.05$ ) and 2.1-fold ( $P<0.01$ ), and inversely, vimentin protein was increased 1.2- and 1.7-fold ( $P<0.05$ ) at 24 and 48 h in BxPC-3 cells, respectively. (F) E-cadherin mRNA was decreased 2.2-fold ( $P<0.01$ ) and 6.8-fold ( $P<0.01$ ) at 24 and 48 h in BxPC-3 cells, respectively. (G) Vimentin mRNA was increased 1.3- and 3.9-fold ( $P<0.01$ ) at 24 and 48 h in BxPC-3 cells, respectively. GAPDH was used as the loading control, and  $\beta$ -actin was used as the housekeeping gene. Results are expressed as the magnitude of relative expression (means  $\pm$  SEM) compared with the control, from three independent experiments. \* $P<0.05$ , \*\* $P<0.01$ . n.c., negative control; SFM24hr, serum-free medium for 24 h; SFM48hr, serum-free medium for 48 h.

vimentin mRNA level due to hypoxia at 24 and 48 h, respectively (Fig. 1E), and in BxPC-3 cells, the vimentin mRNA level was increased 1.7- and 1.9-fold ( $P<0.05$ ) due to hypoxia at 24 and 48 h, respectively (Fig. 1H).

*Under serum starvation, E-cadherin protein and mRNA expression were significantly decreased, while vimentin was increased.* In the next experiment, we tested the effect of serum starvation on E-cadherin and vimentin protein and mRNA expression in pancreatic cancer cells. For serum starvation, Colo-357 and BxPC-3 cells were cultured with 0.5% FCS; this was the second model of pancreatic cell microenvironment change. Due to serum starvation, in Colo-357 cells, E-cadherin protein was decreased 1.5-fold ( $P<0.05$ ) and 4.3-fold ( $P<0.01$ ) at 24 and 48 h, respectively (Fig. 2A), and in BxPC-3 cells, E-cadherin protein was decreased 1.6-fold ( $P<0.05$ ) and 2.1-fold ( $P<0.01$ ) at 24 and 48 h, respectively (Fig. 2E). Meanwhile, in Colo-357 cells, E-cadherin mRNA was decreased 1.4-fold ( $P<0.01$ ) and 5.9-fold ( $P<0.01$ ) at 24 and 48 h, respectively (Fig. 2B), and in BxPC-3 cells, E-cadherin mRNA was decreased 2.2-fold ( $P<0.01$ ) and 6.8-fold ( $P<0.01$ ) at 24 and 48 h, respectively (Fig. 2F). Conversely, in Colo-357 cells, vimentin protein was increased 2.4-fold ( $P<0.05$ ) and 2.8-fold ( $P<0.05$ ) at 24 and 48 h, respectively (Fig. 2C), and in BxPC-3 cells, vimentin protein was

increased 1.2- and 1.7-fold ( $P<0.05$ ) at 24 and 48 h, respectively (Fig. 2E). In accordance, in Colo-357 cells, vimentin mRNA was increased 1.4-fold ( $P<0.05$ ) and 1.6-fold ( $P<0.05$ ) at 24 and 48 h, respectively (Fig. 2D), and in BxPC-3 cells, vimentin mRNA was increased 1.3- and 3.9-fold ( $P<0.01$ ) at 24 and 48 h, respectively (Fig. 2G).

*Following exposure to TGF- $\beta$  induction, E-cadherin protein and mRNA expression are obviously upregulated, while vimentin is downregulated.* Subsequently, we detected the effect of TGF- $\beta$  induction on E-cadherin and vimentin protein and mRNA expression in pancreatic cancer cells. This was the third model of pancreatic cancer cell microenvironment, and TGF- $\beta$  was used as an inducer of EMT. Due to TGF- $\beta$  induction, in Colo-357 cells, E-cadherin protein was decreased 2.1-fold ( $P<0.05$ ) and 3.2-fold ( $P<0.01$ ) at 24 and 48 h, respectively (Fig. 3A), and in BxPC-3 cells, E-cadherin protein was decreased 1.6-fold ( $P<0.05$ ) and 1.7-fold ( $P<0.05$ ) at 24 and 48 h, respectively (Fig. 3C). In Colo-357 cells, E-cadherin mRNA was decreased 1.7-fold ( $P<0.05$ ) and 2.8-fold ( $P<0.01$ ) at 24 and 48 h, respectively (Fig. 3B), and in BxPC-3 cells, E-cadherin mRNA was decreased 2.9-fold ( $P<0.01$ ) and 3.8-fold ( $P<0.01$ ) at 24 and 48 h, respectively (Fig. 3D). On the contrary, in Colo-357 cells, vimentin protein was increased 1.8-fold ( $P<0.05$ ) and 1.7-fold ( $P<0.05$ ) at 24 and 48 h,

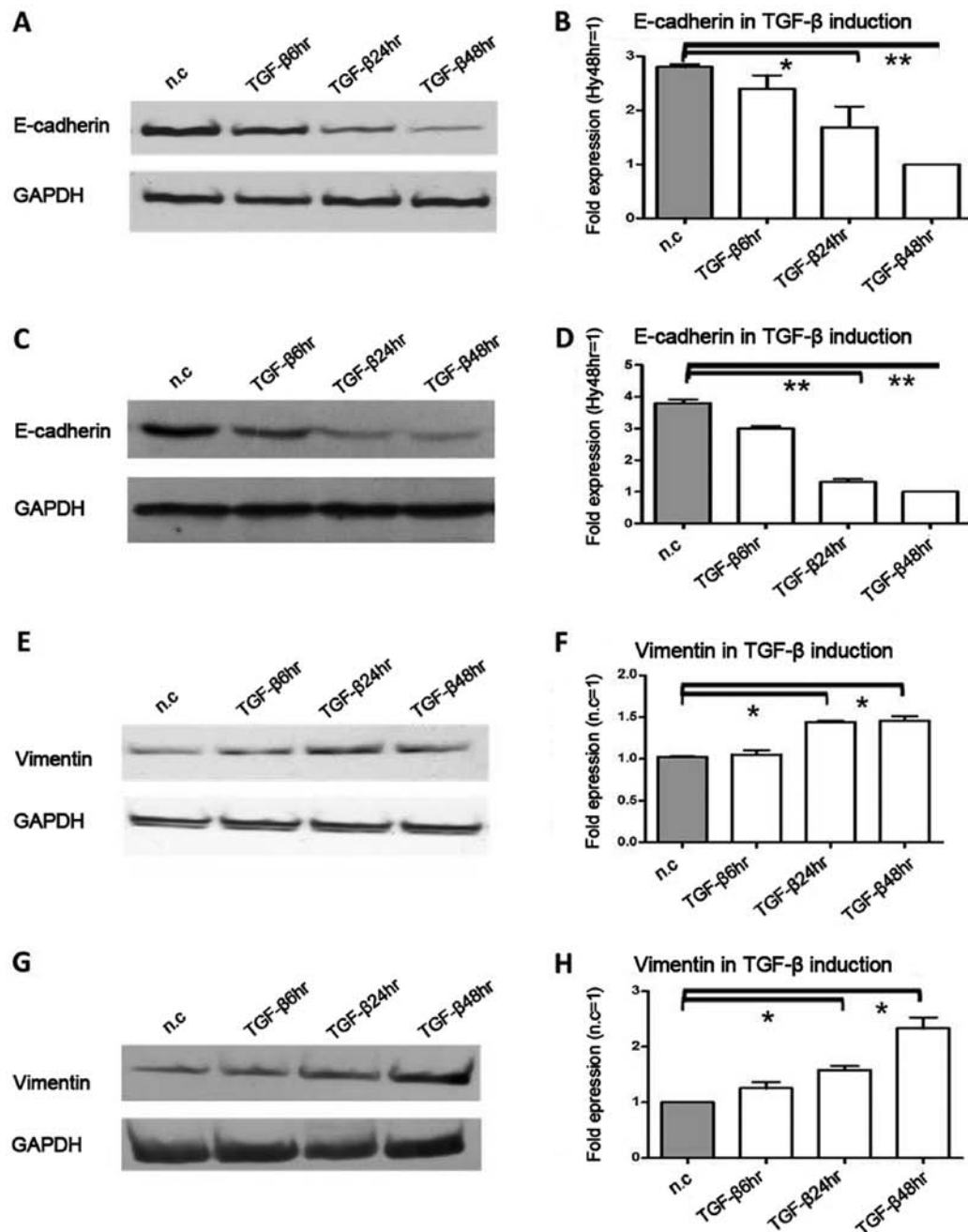


Figure 3. The protein and mRNA expression changes in E-cadherin and vimentin under TGF-β induction, Pancreatic cancer cells Colo-357 and BxPC-3 were incubated with TGF-β (terminal concentration 10 ng/ml) for 6, 24 and 48 h. (A) E-cadherin protein was decreased 2.1-fold ( $P<0.05$ ) and 3.2-fold ( $P<0.01$ ) at 24 and 48 h in Colo-357 cells, respectively. (B) E-cadherin mRNA was decreased 1.7-fold ( $P<0.05$ ) and 2.8-fold ( $P<0.01$ ) at 24 and 48 h in Colo-357 cells, respectively. (C) E-cadherin protein was decreased 1.6-fold ( $P<0.05$ ) and 1.7-fold ( $P<0.05$ ) at 24 and 48 h in BxPC-3 cells, respectively. (D) E-cadherin mRNA was decreased 2.9-fold ( $P<0.01$ ) and 3.8-fold ( $P<0.01$ ) at 24 and 48 h in BxPC-3 cells, respectively. (E) Inversely, vimentin protein was increased 1.8-fold ( $P<0.05$ ) and 1.7-fold ( $P<0.05$ ) at 24 and 48 h in Colo-357 cells, respectively. (F) Vimentin mRNA was increased 1.5-fold ( $P<0.05$ ) and 1.6-fold ( $P<0.05$ ) at 24 and 48 h in Colo-357 cells, respectively. (G) Vimentin protein was increased 1.2- and 1.5-fold ( $P<0.05$ ) at 24 and 48 h in BxPC-3 cells, respectively. (H) Vimentin mRNA was increased 1.4-fold ( $P<0.05$ ) and 2.3-fold ( $P<0.05$ ) at 24 and 48 h in BxPC-3 cells, respectively. GAPDH was used as the loading control, and β-actin was used as the housekeeping gene. Results are expressed as the magnitude of relative expression (means  $\pm$  SEM) compared with the control, from three independent experiments. \* $P<0.05$ , \*\* $P<0.01$ . n.c., negative control; TGF-β6hr, TGF-β induction for 6 h; TGF-β24hr, TGF-β induction for 24 h; TGF-β48hr, TGF-β induction for 48 h.

respectively (Fig. 3E), and in BxPC-3 cells, vimentin protein was increased 1.2- and 1.5-fold ( $P<0.05$ ) at 24 and 48 h, respectively (Fig. 3G). Similarly, in Colo-357 cells, vimentin mRNA was increased 1.5-fold ( $P<0.05$ ) and 1.6-fold ( $P<0.05$ ), respectively (Fig. 3F), and in BxPC-3 cells, vimentin mRNA was increased 1.4-fold ( $P<0.05$ ) and 2.3-fold ( $P<0.05$ ), respectively (Fig. 3H).

*Location and varying expression intensity of E-cadherin in pancreatic cancer tissues.* In order to investigate the expression of E-cadherin in pancreatic tissues, immunohistochemistry was performed in consecutive sections of 52 pancreatic ductal adenocarcinoma (PDAC) tissues. E-cadherin immunoreactivity showed varying intensity in different pancreatic cancer tissues, and was clearly found in cell membrane staining. For

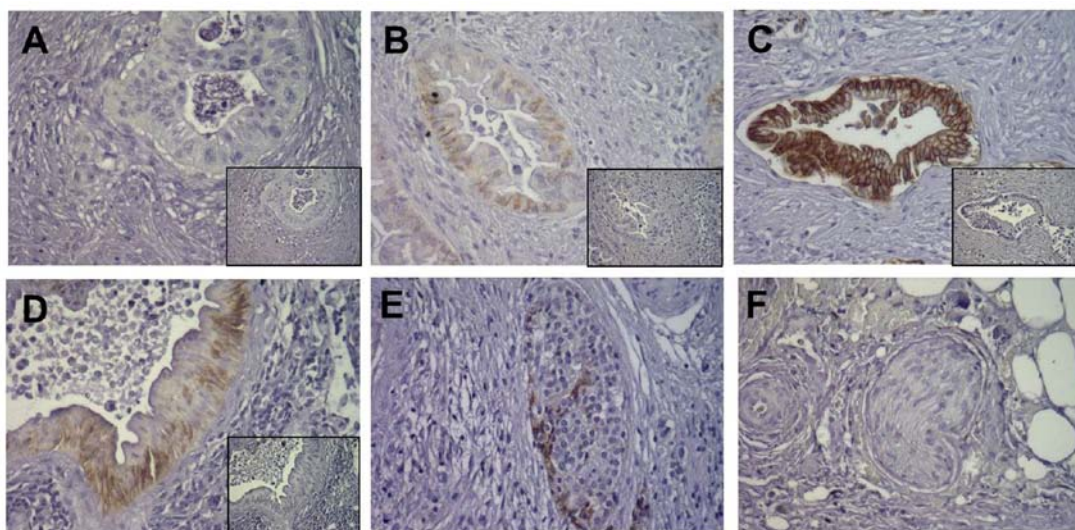


Figure 4. E-cadherin location and expression in pancreatic cancer tissues (magnifications, x100). Immunohistochemistry was performed with E-cadherin (1:200) anti-rabbit antibodies. (A) Localization of E-cadherin in plasmalemma of pancreatic cancer cells showed weak staining. (B) Moderate expression of E-cadherin shown by plasmalemma staining of cancer cells. (C) Strong expression of E-cadherin shown by plasmalemma staining of cancer cells. (D) Strong expression of E-cadherin by plasmalemma staining of PanIN lesion. (E) Strong expression in some pancreatic islet cells. (F) No staining in pancreatic nerves.

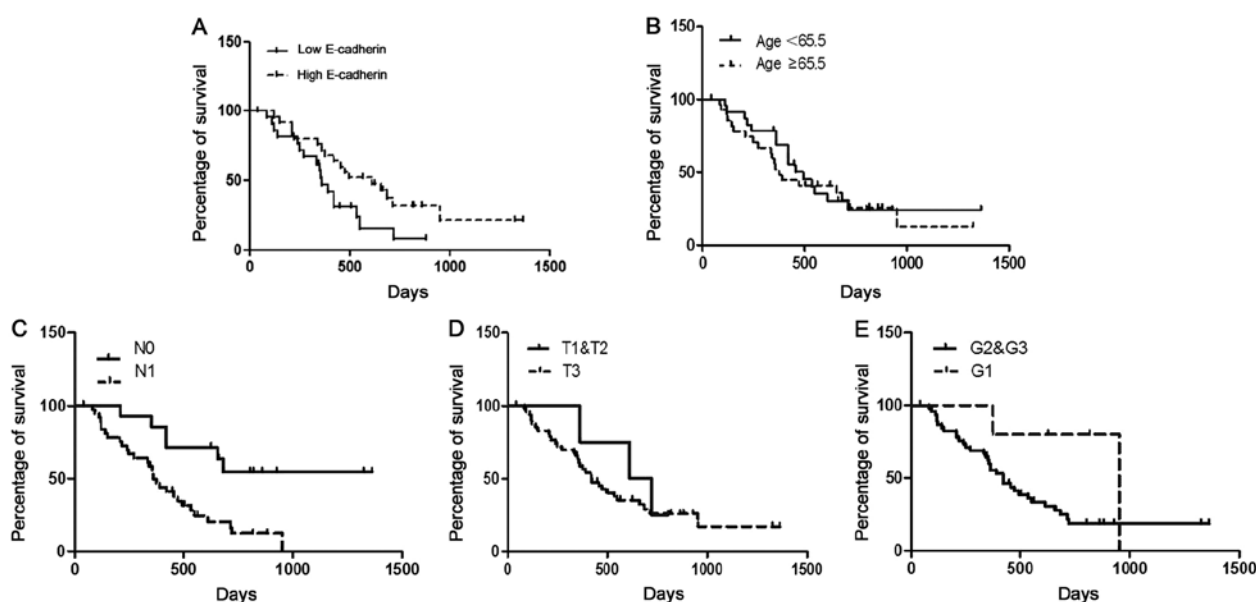


Figure 5. Kaplan-Meier and log-rank analyses of prognostic factors assessed in a multivariable analysis using a Cox proportional hazards model. (A) Patients with higher levels of E-cadherin expression [n=22, median survival=22.9 (18.9-26.8) months] had a significantly longer survival time compared to those with lower E-cadherin expression [n=30, median survival=14.1 (9.1-19.1) months,  $P<0.01$ ]. (B) There was no significant difference in survival time between patients of age  $\geq 65.5$  years [n=19, median survival=18.2 (12.8-23.7) months] and patients  $<65.5$  years [n=23, median survival=21.2 (14.9-27.4) months,  $P>0.05$ ]. (C) Patients with lymph node metastasis [n=38, median survival=14.2 (11.2-17.3) months] had a significantly shorter survival time compared to those without lymph node metastasis [n=14, median survival=31.9 (23.6-40.1) months,  $P<0.01$ ]. (D) There was no significant difference in survival time between patients of T1&T2 [n=4, median survival=20.8 (15.3-26.2) months] and patients of T3 [n=48, median survival=19.2 (14.7-23.8) months,  $P>0.05$ ]. (E) There was no significant difference in survival time between patients of G1&G2 [n=5, median survival=27.9 (15.2-37.4) months] and patients of G3 [n=47, median survival=18.6 (14.2-22.9) months,  $P>0.05$ ]. Median values were taken as the cut-off limits when the two groups were compared. Survival analyses were performed using the Kaplan-Meier method for the estimation of event rates, and a log-rank test was used for survival comparisons between patient groups. The P-value was 5%.

E-cadherin, some cancer cells had weak and moderate expression in 30 cases (57.7%, Fig. 4A and B), while some cancer cells were strongly positive in 22 cases (42.3%, Fig. 4C). In pancreatic PanIN lesions, E-cadherin was strongly expressed (Fig. 4D). In addition, E-cadherin was also strongly expressed in pancreatic islet cells (Fig. 4E), whereas there was no E-cadherin staining in pancreatic nerves (Fig. 4F).

*Correlation of E-cadherin expression in pancreatic cancer with patient survival.* Based on differential E-cadherin expression levels in PDAC tissues, we further divided the PDAC patients into E-cadherin-weak and E-cadherin-strong expression groups. The demographics and the association between E-cadherin expression and clinicopathological features are displayed in Table I. The correlation analysis results indicated

Table II. Univariable and multivariable analysis of prognostic factors.

Variable	Comparator	N	Univariable analysis				Multivariable analysis		
			Median survival (95% CI) in months	Log-rank	P-value		HR	95% CI	P-value
Median age (years)	≥65.5 vs. <65.5	19/23	18.2 (12.8-23.7) vs. 21.2 (14.9-27.4)	0.191	0.662		0.813	0.149-0.763	0.367
Sex	M/F	26/26	20.48 (14.2-26.8) vs. 18.4 (12.9-23.8)	0.409	0.523		0.468	0.388-1.579	0.494
T-status	T3 vs. T1 & T2	48/4	19.2 (14.7-23.8) vs. 20.8 (15.3-26.2)	0.384	0.538		0.277	0.205-2.496	0.599
N-status	N1 vs. N0	38/14	14.2 (11.2-17.3) vs. 31.9 (23.6-40.1)	9.592	0.004		9.445	0.077-0.567	0.002
Grade	G1 vs. G2 & G3	5/47	27.9 (15.2-37.4) vs. 18.6 (14.2-22.9)	3.153	0.095		2.801	0.797-17.735	0.094
E-cadherin score	≤4 vs. >4	30/22	14.1 (9.1-19.1) vs. 22.9 (18.9-26.8)	11.235	0.001		6.810	0.149-0.763	0.009

CI, confidence interval; HR, hazard ratio.

that the expression level of E-cadherin was not related to patient sex, age, tumor-node-metastasis (TNM) staging or histological grade. Consistently, patients with higher levels of E-cadherin expression [n=22, median survival=22.9 (18.9-26.8) months] had significantly longer survival times compared to those with lower E-cadherin expression [n=30, median survival=14.1 (9.1-19.1) months,  $P<0.01$ ] (Fig. 5A). The prognostic value of E-cadherin expression was assessed by multivariable analysis using a Cox proportional hazards model (Table II). Based on the factors analyzed, the results revealed that the expression of E-cadherin [HR=6.810, 95% confidence interval (CI), 0.149-0.763,  $P<0.01$ ] and the occurrence of lymph node metastasis [hazard ratio (HR)=9.445, 95% CI, 0.077-0.567,  $P<0.01$ ] were independent prognostic factors (Fig. 5C). The other related prognostic factors (age, T-status, grade), did not appear to impact the prognosis independently (Table II and Fig. 5B, D and E).

## Discussion

It is well known that a hypoxic environment is a common feature of several solid tumors. While normal tissues generally receive an oxygen ( $O_2$ ) pressure of 30-50 mmHg, the pressure drops to below 2.5 mmHg in up to 50-60% of locally advanced solid tumors (14). When  $O_2$  levels were measured in patients with various solid tumors using  $pO_2$  histography, pancreatic cancer was found to be the most hypoxic, and intraoperative  $pO_2$  measurements of seven resectable pancreatic tumors further showed the hypoxic microenvironment (15,16). Several mechanisms contribute to the hypoxic milieu of PDAC. The major mechanism underlying reduced tumor oxygenation is the insufficient and aberrant vasculature that cannot deliver the necessary blood supply to all parts of the tumor tissue. In addition, stromal cells in PDAC may contribute to this hypoxia both by amplifying the production of antiangiogenic substances or by physically compressing the capillaries through extracellular matrix deposition in the periacinar spaces. Importantly, hypoxia is an important activator of pancreatic stellate cells (PSCs), the major fibroblastic cells of the pancreas, which perpetuate the vicious cycle of hypoxia and fibrosis (17,18). It is likely that the already fibrotic and hypovascular microenvironment of pancreatic cancer is one of the reasons for the failure of antiangiogenic therapies in pancreatic cancer in the clinical setting. Overall, reduced microvessel density, nutrient deprivation and extracellular matrix deposition create a hypoxic setting for pancreatic cancer cells.

Hypoxia induces EMT and plays a major role in the metastatic phenotype of PDAC cells (19-22). In EMT, cancer cells acquire mesenchymal features, such as dissolution of adhesion to the extracellular matrix and loss of cell polarity, which result in their transition into invasive cells. Moreover, EMT promotes the survival of cancer cells in the blood and metastatic sites by endowing them with stem-cell-like features and inhibiting their apoptosis. Furthermore, cells that have already undergone EMT migrate at the forefront of the invading cancer, creating tracks that other cancer cells can exploit. EMT is one important molecular mechanism underlying early invasion and metastasis in PDAC (23,24).

E-cadherin is a classic cadherin (calcium-dependent adhesion molecule) family member, with key roles in cell growth



and differentiation, apoptosis and morphological changes in normal tissues and cells. E-cadherin protein promotes the formation of stable intercellular connections between adjacent cells, promotes epithelial cell adhesion, and maintains the integrity of tissue structure and function (25-27). In addition, E-cadherin can inhibit tumor cell secretion of matrix metalloproteinases (MMPs). Therefore, the loss of E-cadherin protein expression decreases the mutual adhesion force between tumor cells, which can then break through the extracellular matrix and basement membrane, detach from the primary tumor and invade and metastasize (28,29). As a very sensitive EMT marker, the expression of E-cadherin is often related to the infiltration and undifferentiated phenotype of tumor cells, and it is closely related to invasion and metastasis of early tumor cells (30,31).

Throughout cancer development, the expression of the mesenchymal marker vimentin is increased. Cancer cells often have altered cell surface molecules, and vimentin is an important molecular marker of mesenchymal cells. Although mature epithelial cells show no vimentin expression, during epithelial cell migration, vimentin can be re-expressed (32). Our experimental results showed that the expression of E-cadherin was decreased gradually over time with microenvironmental changes (including lack of serum and anaerobic environment), suggesting that microenvironmental changes can downregulate E-cadherin to inhibit the epithelial phenotype of pancreatic cancer cells. Decreases in E-cadherin induced by changes to the tumor microenvironment may be involved in the invasion and metastasis of pancreatic cancer cells. In contrast, alterations in the microenvironment can significantly promote the expression of vimentin in pancreatic cancer cells, further demonstrating that changes in the microenvironment of pancreatic cancer cells lead to EMT. In addition, HIF-1 $\alpha$  was used as a marker for the successful creation of an anaerobic environment, and its protein expression was upregulated, suggesting that the anaerobic state was achieved in the microenvironment surrounding the pancreatic cancer cells.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a multifunctional cytokine that participates in the autocrine and paracrine regulation of a variety of biological functions in cells, including the inhibition of epithelial cell, immune cell and hematopoietic cell proliferation, the promotion of angiogenesis, and the induction of fibroblast differentiation from normal epithelial cells, a process of transformation that is closely related to E-cadherin expression (33,34). Our study applied TGF- $\beta$  as an EMT-inducing factor. The results showed that TGF- $\beta$  addition induced changes to the levels of E-cadherin and vimentin mRNA in pancreatic cancer cells that were consistent with those that occurred under serum starvation and in a hypoxic environment, which further confirmed the interactions of TGF- $\beta$ , E-cadherin and vimentin in EMT in pancreatic cancer cells.

Based on the results of immunohistochemistry, E-cadherin expression levels were significantly reduced in most pancreatic cancer samples. In our study, we analyzed 30 pancreatic cancer tissues from 50 patients with lower E-cadherin expression (58%), and the Kaplan-Meier and log-rank analyses showed that the expression level of E-cadherin and the survival time of patients with pancreatic cancer were positively correlated. It is worth noting that precancerous lesions such

as PanINs and tubular complexes have abundant E-cadherin expression. This result also indirectly shows that the changes in expression of E-cadherin, which occur throughout the transformation of benign pancreatic diseases to pancreatic cancer, occur simultaneously with EMT. Therefore, research into the mechanism of this occurrence and the development of pancreatic cancer is important.

Results from the multivariable analysis of the Cox proportional hazards model showed that the expression of E-cadherin and the occurrence of lymph node metastasis both were independent prognostic factors of pancreatic cancer. G staging showed a trend towards correlation with patient overall survival, but there was no statistical significance, and an increased sample size would be needed to confirm these findings. In summary, the characteristics of the pancreatic cancer microenvironment can induce pancreatic cancer cell EMT, furthering the cell invasion and metastasis capacities.

Further research efforts should focus on how to effectively address the microenvironmental conditions (i.e., the lack of serum and occurrence of a hypoxic environment) of pancreatic cancer cells to inhibit their malignancy. It is well known; the tumor cell microenvironment is essential for the generation of EMT in pancreatic cancer. The pancreatic cancer cells, extracellular matrix cells and related cytokines are the most important components of the pancreatic cancer microenvironment (24). In order to investigate the relationship *in vivo* between the microenvironment and EMT, we can conduct primary cultures of pancreatic cancer cells *in vitro* from the surgical resection of pancreatic cancer tissues. We can also extract the extracellular matrix cells (the activated stellate cells) *in vitro*, and then combine pancreatic cancer cells and stellate cells and transplant into the pancreas of mice. By successfully establishing a patient-derived xenograft model (PDX), we can continually develop a series of studies concerning the microenvironment and EMT *in vivo*.

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## Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

## Authors' contributions

YZ conceived and designed the study. WW, LD, BZ and JL performed the experiments. WW and LD wrote the paper. WW and LD reviewed and edited the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the



accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

All experimental protocols were approved by the Peking Union Medical College Hospital Ethics Committee (Beijing, China).

### Patient consent for publication

Not applicable.

### Competing interests

The authors state that they have no competing interests.

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