Epithelial - to - Mesenchymal Transition Predicts Prognosis in Clinical Gastric Cancer

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Synopsis: EMT status, as determined for the first time by a V/E ratio, was found to be a critical prognostic factor in gastric cancer. Furthermore, Zeb-1 could be the most important regulator for EMT in this disease and could also be a prognostic factor.

ABSTRACT

Background: Epithelial-to-mesenchymal transition **(**EMT) is considered to play an important role in cancer invasion and metastasis.

Methods: The mRNA levels of an epithelial marker (E-cadherin), mesenchymal marker (vimentin) and Zeb-1 were measured in 11 gastric cancer cell lines. Functional analysis was performed using Zeb-1 knockdown. EMT status of 116 gastric cancer patients was determined by calculating the vimentin/E-cadherin mRNA expression ratio in cancerous tissue and the correlation between EMT status, clinicopathological factors, prognosis and Zeb-1 were analyzed.

Results: Cell lines were classified as epithelial or mesenchymal. Zeb-1 expression was significantly correlated with the mesenchymal phenotype. Treatment with Zeb-1 siRNA also reduced the capacity to proliferate, migrate and invade. Patients were classified as epithelial or mesenchymal by V/E ratio (vimentin/E-cadherin ratio) and as Zeb-1 low or high expression group. The mesenchymal group was significantly associated with diffuse type cancer and stage IV. On multivariate analysis, the EMT status (mesenchymal group) was an independent prognostic factor (P=0.022). There was a significant correlation between the V/E ratio and Zeb-1 expression (r=0.73). Patients in Zeb-1 high group had significantly poorer survival than those in low group (P=0.0071).

Conclusions: EMT is a critical prognostic factor for gastric cancer. Zeb-1 might be a promising therapeutic target.

Key Words: gastric cancer; epithelial-to-mesenchymal transition; Zeb-1; prognosis

INTRODUCTION

Gastric cancer is one of the most common malignant tumors worldwide, ranking fourth among all malignant tumors, and the second leading cause of cancer-related death [1]. Approximately one-third of gastric cancer patients have advanced cancer with distant metastasis at the time of diagnosis [2], and the prognosis of these patients remains poor.

During epithelial-to-mesenchymal transition (EMT), epithelial cells undergo a phenotypic switch to form mesenchymal cells that are similar in appearance to fibroblasts [3,4]. EMT, characterized by a gain of mesenchymal cell markers and a loss of epithelial markers [5,6], is a process whereby cells acquire molecular alterations that facilitate cell motility and invasion [7]. This phenomenon is reactivated during the progression of numerous cancers including cutaneous, prostatic, mammary, hepatic, gastric, pancreatic and colorectal [8-13].

Several EMT-inducing transcription factors, including Twist, Snail, Slug, Zeb-1 and Zeb-2 have been actively studied and are known to induce dramatic spreading and morphological changes in cancer cells through suppression of E-cadherin, and induction of mesenchymal markers [14,15]. Some studies have advocated the importance of EMT-inducing regulators, such as Snail, Slug, Twist, Zeb-1 and Zeb-2 in gastric cancer [16-20]; however, most studies have been conducted *in vitro* and the implication of EMT *in vivo* remains unclear.

In the current study, surgical specimens from gastric cancer patients in our department were utilized to examine the clinical implication of EMT. Attempts were made to determine the EMT status of each gastric cancer specimen and the correlation between the EMT status and clinicopathological factors and prognosis was evaluated [21].

Furthermore, the most important transcription factor for promoting EMT was analyzed not only *in vitro* but also *in vivo*. Novel insights into the mechanistic pathways for EMT could provide new and effective treatment modalities for this disease.

MATERIALS AND METHODS

Cell Lines and Cultures

Human gastric cancer cell lines, (GCIY, MKN28, MKN45 and MKN74) were obtained from RIKEN Cell Bank (Tsukuba, Japan). The gastric cancer cell lines NUGC-2 and NUGC-4 were established and maintained at the Department of Surgery II, Nagoya University Graduate School of Medicine. AZ521 and MKN1 were provided by Japanese Cancer Research Resource Bank (Tokyo, Japan). KATOIII and N87 were obtained from ATCC (Manassas, VA, USA). SC-6-JCK were established and kindly donated by the Department of Surgery, Research Institute for Microbial Disease, Osaka University and Central Institute for Experimental Animals. All cells were grown in DMEM (Wako Pure Chemical Industries, Ltd. Japan) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, N.Y.), and incubated at 37℃ in a humidified chamber supplemented with 5% CO_2.

Patients and Specimens

A total of 116 consecutive patients underwent surgery for gastric cancer from November 2001 to January 2008 at Nagoya University Hospital. The mean age was 64 years (range, 21-84). 83 patients were male, 33 patients were female. Patients were staged according to the UICC cancer staging criteria for gastric cancer (7th edition, 2009). A total of 12 patients had stage IA disease, 10 had Stage IB disease, 7 had stage IIA

disease, 13 had stage IIB disease, 9 had stage IIIA disease, 14 had stage IIIB disease, 11 had stage IIIC disease and 40 had stage IV disease. The median follow-up period was 37 months (range: 1-117 months). This study was approved by Ethics Committee of the hospital and the signed informed consent was obtained from all patients.

Real-time Quantitative PCR Analysis

Total RNA from cell lines and human gastric tissues was isolated using ISOGEN (NIPPON GENE, Japan) according to the manufacture's protocol. Real-time quantitative PCR analysis was performed as described previously [22]. PCR primers of each gene are as follows: E-cadherin: 5- GAAGGTGACAGAGCCTCTGGAT-3 (forward) and 5-CATTCCCGTTGGATGACACA-3 (reverse), which amplify a 79 bp product, Vimentin: 5-AAAACACCCTGCAATCTTTCAGA-3 (forward) and 5-GATTCCACTTTGCGTTCAAGGT-3 (reverse), which amplify a 78 bp product, Twist: 5-GTCCGCAGTCTTACGAGGAG-3 (forward) and 5-CCAGCTTGAGGGTCTGAATC-3 (reverse), which amplify a 159 bp product, Slug: 5-CTTTTTCTTGCCCTCACTGC-3 (forward) and 5-GCTTCGGAGTGAAGAAATGC-3 (reverse), which amplify a 224 bp product, Snail: 5-ACCCCACATCCTTCTCACTG-3 (forward) and 5-TACAAAAACCCACGCAGACA-3 (reverse), which amplify a 217 bp product, Zeb-1: 5-TGCACTGAGTGTGGAAAAGC-3 (forward) and 5-TGGTGATGCTGAAAGAGACG-3 (reverse), which amplify a 237 bp product, Zeb-2: 5-GAGTGGCCGAAAGAGATCAG-3 (forward) and 5-AGTTTTGGCCAGAAATGGTG-3 (reverse), which amplify a 181 bp product.

Western Blotting Analysis

Western blotting analysis was performed as described previously [22]. The following primary antibodies were used: rabbit anti-E-cadherin, rabbit anti-vimentin and mouse anti-TCF8/Zeb-1 (1:1000, Cell Signaling Technology, Beverly, MA, respectively).

Transfection of Zeb-1 Short Interfering RNA

MKN1 cells were plated in 6-well plates at a density of 2.5 $x10^4$ cells/ml. Cells were transiently transfected the next day with either 30 nM predesigned short interfering RNA (siRNA) targeting Zeb-1 or control siRNA (Ambion) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 72h, the expression of protein levels of Zeb-1, E-cadherin and vimentin were analyzed by western blotting.

Cell Proliferation, Migration, and Invasion Assays

Cell proliferation assays were carried out by cell counting in 6-well plates at 24, 48, 72 and 96 h after transfection. Wound-healing assays were performed 48 h after transfection in 6-well plates. A wound was made through the cells with a 1000μl micropipette tip. The area of migration was measured by ImageJ software. Cell invasion was assessed by using Matrigel Invasion Chambers (BD Biosciences). 48h after transfection, cells were plated in transwell chambers pre-coated with Matrigel Invasion Chamber medium. After incubation, the non-invading cells were removed with cotton swabs. The invasive cells that attached to the lower surface of the membrane were stained with Diff-Quick Stain kit (Sysmex corporation). The number of invasive cells on the lower surface of the membrane was randomly counted in five vision fields under a microscope (400X).

Statistical Analysis

Differences in the numerical data between two groups were evaluated using Fisher's exact test or χ^2 test. Overall survival rates were calculated using the Kaplan-Meier

method, and the difference in survival curves was analyzed using the log-rank test. Independent prognostic factors were analyzed with the Cox proportional hazards regression model in a stepwise manner. The associations between V/E ratio and Zeb-1 mRNA expression were analyzed by Pearson correlation coefficient. Data are expressed as mean ± SD. The presence of a statistically significant difference was denoted by $P < 0.05$. Data were analyzed using JMP version 9 software (JMP, SAS Institute, Cary, NC).

RESULTS

Characterization of EMT in Human Gastric Cancer Cell Lines

The expression of E-cadherin and vimentin was assessed in 11 human gastric cancer cell lines to determine their extent of EMT. Seven of the cell lines (N87, NUGC2, MKN45, NUGC4, KATOIII, MKN74 and SC-6-JCK) were classified as epithelial, because these cell lines express E-cadherin at high levels and they lack vimentin expression. Conversely, four of the cell lines (GCIY, MKN28, AZ521 and MKN1) were considered to be mesenchymal, because these cell lines express vimentin at high levels and they lack E-cadherin expression (data not shown).

Transcription Factors for EMT in Human Gastric Cancer Cell Lines

Transcription factors (Twist, Slug, Snail, Zeb-1, Zeb-2) were assessed in each of the gastric cancer cell lines and the quantitative real-time PCR results showed that Zeb-1 mRNA expression was strongly associated with vimentin expression. In other words, high expression of Zeb-1 was significantly correlated with mesenchymal phenotype (data not shown). On the other hand, expression levels of Twist, Slug, Snail and Zeb-2 were

not associated with mesenchymal phenotype (data not shown). These results indicate that Zeb-1 may play a key role in the EMT process for gastric cancer cell lines ($r = 0.71$, $P = 0.015$) (data not shown).

Effect of Zeb-1 Expression in Proliferation, Migration and Invasion

To further verify the relation of Zeb-1 with EMT in gastric cancer, Zeb-1 siRNA was transfected into mesenchymal MKN1 cells and the effects on proliferation, migration and invasion were evaluated. The expression level of Zeb-1 protein and mRNA was significantly decreased in MKN1 cells after treatment with Zeb-1 siRNA, and subsequently, the expression of E-cadherin protein was significantly increased while vimentin was decreased. These results demonstrated that EMT could be regulated by Zeb-1 (Fig. 1A).

Next, the proliferation ability was examined in MKN1 cells using Zeb-1 siRNA. Zeb-1 siRNA significantly reduced cell proliferation compared to controls ($P < 0.05$) (Fig.1B). Cell migration ability was examined with a scratch wound-healing assay, and the Zeb-1 siRNA group was distinctively less migratory than the controls ($P < 0.05$) (Fig. 1C). Likewise, invasiveness was significantly reduced in the Zeb-1 siRNA group ($P < 0.05$) (Fig. 1D). Thus, these results demonstrated that the knockdown of Zeb-1 as a transcription factor could regulate EMT and could reduce proliferation, migration and invasion in gastric cancer cells.

Clinical Implication of EMT in Gastric Cancer Patients

EMT status was determined using a V/E ratio (vimentin mRNA expression divided by E-cadherin mRNA expression in cancerous tissues) in clinical gastric cancer specimens. Patients with a V/E ratio < 1.63 (mean) were assigned to the epithelial group ($n = 86$),

whereas those with a V/E ratio ≥ 1.63 were assigned to the mesenchymal group (n = 30). Table I showed the correlation between clinicopathological variables and EMT status, which revealed that the mesenchymal group was significantly associated with pathological diffuse type cancer (P = 0.0002) and stage IV disease (P = 0.046).

The patients in the mesenchymal group also had significantly poorer survival than those in epithelial group (P = 0.013), and the 5-year survival rate of patients in the mesenchymal group (34.2%) was significantly lower than that of patients in the epithelial group (55.3%) (Fig. 2A).

Clinical Significance of Zeb-1 Expression in Gastric Cancer Patients

We performed a similar analysis for Zeb-1 expression. Patients with low Zeb-1 mRNA expression (< 0.0217 (mean)) were assigned to the Zeb-1 low group (n = 84), whereas those with high expression (≥ 0.0217) were assigned to the Zeb-1 high group (n = 32). Table II showed that Zeb-1 high group was significantly associated with age (≤ 63), pathological diffuse type and number of lymph node metastasis (n ≥ 16), (P = 0.036, P = 0.035, P = 0.018, respectively).

In addition, patients in the Zeb-1 high group had significantly poorer survival than those in the Zeb-1 low group (P = 0.0071), and the 5-year survival rate of patients with high Zeb-1 expression (37.4%) was significantly lower than that of patients with low Zeb-1 expression (55.6%) (Fig. 2B).

There was also a very significant correlation between V/E ratio and Zeb-1 mRNA expression (r = 0.73, P < 0.0001) (Fig. 2C). This result further indicates that Zeb-1 expression was an essential mediator of EMT in gastric cancer.

Association Between EMT Status and Prognosis in Gastric Cancer.

The association between EMT status and prognosis in gastric cancer patients was evaluated by multivariate analysis. Univariate analysis showed that tumor size (≥ 65mm), pathological type (diffuse type), T classification (pT3/T4), vessel involvement, lymphatic vessel involvement, lymph node metastasis, peritoneal metastasis and EMT status (mesenchymal) were significantly associated with poor survival. On multivariate analysis, tumor size (≥ 65mm) (P = 0.027), T classification (pT3/4) (P = 0.0098), peritoneal metastasis (P = 0.0028) and EMT status (mesenchymal) (P = 0.022) were independent prognostic factors for gastric cancer patients (Table III).

DISCUSSION

The mRNA expression of epithelial markers, such as E-cadherin, decrease whereas those of mesenchymal markers, such as vimentin, increase during EMT [10,23,24]. From a clinical standpoint, the loss of E-cadherin has been reported to associate with poor clinical outcome in several types of cancers [25-28], including gastric cancer [16,17]. On the other hand, vimentin expression in epithelial cancer cells has been indicated to associate with metastasis and poor survival in several cancers [29,30].

To better understand the impact of EMT on the prognosis of gastric cancer patients, we measured not only an epithelial maker (E-cadherin) but also a mesenchymal marker (vimentin) in this study. In this regard, this is the first report characterizing gastric cancer cell lines and patient specimens as epithelial or mesenchymal based on their extent of E-cadherin and vimentin expression [31,32]. When the EMT status was determined using a V/E ratio [21], patients could be divided into epithelial or mesenchymal groups. Patients

in the mesenchymal group had significantly poorer survival than those in the epithelial group. Furthermore, the EMT status was identified to be an independent prognostic factor in our 116 resected surgical specimens. In a previously reported study, vimentin expression was significantly higher in diffuse type tumors than in intestinal type tumors and was significantly higher in patients with recurrent or distant metastatic disease, and E-cadherin expression was significantly associated with intestinal type cancer [30]. These findings are consistent with and strongly support our results, and our way to predict the prognosis could be more useful. In this study, the mesenchymal group was significantly associated with pathological diffuse type cancer and stage IV disease. These findings suggest that the EMT status is significantly related to tumor progression and metastasis.

Previous studies have suggested that Zeb-1 is an important mediator of EMT in gastric cancer [19,20]. To further verify the relationship of Zeb-1 with invasion and metastasis in gastric cancer, Zeb-1 siRNA was transfected into MKN1 cells that have a mesenchymal phenotype. Knockdown of Zeb-1 showed that E-cadherin expression was increased, whereas vimentin expression was decreased, further suggesting that the expression of E-cadherin and vimentin in gastric cancer cell could be regulated by Zeb-1. Furthermore, proliferation, migration and invasiveness, which are essential for EMT, were significantly lower in the Zeb-1 siRNA transfected group than in controls. We observed similar results when Zeb-1 siRNA was transfected into two additional mesenchymal cell lines (MKN28 and AZ521). Thus, these results suggest that therapeutic Zeb-1 knockdown could elicit a mesenchymal-to-epithelial transition (MET) and possibly improve prognosis in gastric cancer. However, these results will need to be verified by others in additional cell lines.

Zeb-1 mRNA expression was also measured and found to be significantly associated with shorter overall survival. Furthermore, the Zeb-1 high group was significantly associated with lower age, pathological diffuse type cancer and multiple lymph node metastasis. It was especially noticeable that both the Zeb-1 high and mesenchymal groups correlated with pathological diffuse type and poor overall survival.

In conclusion, EMT status, as determined for the first time by a V/E ratio, was found to be a critical prognostic factor in gastric cancer. Furthermore, Zeb-1 could be the most important regulator for EMT in this disease and could also be a prognostic factor. To improve the survival outcome of gastric cancer, it is necessary to control cancer invasion and metastasis. In this regard, EMT, and in particular Zeb-1, might be a promising therapeutic target for patients with gastric cancer.

Abbreviations

EMT: epithelial-mesenchymal transition

MET: mesenchymal-epithelial transition

PCR: polymerase chain reaction

siRNA: short interfering RNA

SD: standard deviation

HR: hazard ratio

CI: confidence interval

UICC**:** Union for International Cancer Control

TGX: total gastrectomy

PGX: proximal gastrectomy

DGX: distal gastrectomy

PD: pancreatoduodenectomy

PPG: pylorus-preserving gastrectomy

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FIGURE LEGENDS

Fig. 1: A: Zeb-1, E-cadherin and vimentin protein expression in each group of MKN1 cells. Zeb-1 mRNA expression was confirmed in each group of MKN1 cells. **B:** MKN1 cells transfected with Zeb-1 siRNA showed significantly reduced cell proliferation compared with the controls**. C:** MKN1 cells treated with Zeb-1 siRNA showed distinctly less migration than controls. **D:** Diff-Quik staining of MKN1 cells that had passed through the BD Matrigel invasion chamber. Original magnifications were X200. The number of cells at a field of microscope (X400) that had passed through the chamber significantly decreased in Zeb-1 siRNA group. (a: non-transfected control group, b: transfected control siRNA group, c: Zeb-1 siRNA transfected group).

Fig. 2: The survival curves of patients with gastric cancer based on EMT status and Zeb-1 expression level. **A:** The patients in mesenchymal group had significantly poorer survival than those in epithelial group ($P = 0.013$). **B:** Patients in Zeb-1 high-expression group had significantly poorer survival than those in low-expression group ($P = 0.0071$). **C:** V/E ratio (vimentin/E-cadherin ratio) for each tumor sample was plotted against its Zeb-1 expression. Pearson correlation analysis showed a significant correlation between V/E ratio and Zeb-1 expression ($r = 0.73$, $P < 0.0001$).

TABLE I. Correlation between EMT status and Clinicopathological Variables

Variables	Epithelial group (n=86), N (%)	Mesenchymal group (n=30), N (%)	P value
Age			
≥ 64	51 (59.3)	12 (40.0)	0.089
≤ 63	35 (40.7)	18 (60.0)	
Gender			
Male	61 (71.0)	22 (73.3)	1.0
Female	25 (29.0)	8 (26.7)	
Tumor size			
≥ 65 mm	39 (45.3)	17 (56.7)	0.30
65 mm <	47 (54.7)	13 (43.3)	
Pathological type			
Diffuse type	42 (48.8)	26 (86.7)	0.0002*
Intestinal type	44 (51.2)	4 (13.3)	
Pathological T category			
pT3/4	65 (75.6)	25 (83.3)	0.45
pT1/2	21 (24.4)	5 (16.7)	
Vessel involvement			
Yes	48 (55.8)	14 (46.7)	0.40
No	38 (44.2)	16 (53.3)	
Lymphatic vessel involvement			
Yes	74 (86.0)	25 (83.3)	0.77
No	12 (14.0)	5 (16.7)	
Lymph node metastasis			
Yes	18 (20.9)	12 (40.0)	0.13
No	68 (79.1)	18 (60.0)	
Number of lymph node metastasis			
≥ 16	11 (12.8)	6 (20.0)	0.37
< 16	75 (87.2)	24 (80.0)	
Peritoneal metastasis			
Yes	11 (12.8)	6 (20.0)	0.37
No	75 (87.2)	24 (80.0)	
Liver metastasis			
Yes	6 (7.0)	0 (0.0)	0.34
No	80 (93.0)	30 (100.0)	
Stage			
IV	25 (29.1)	15 (50.0)	0.046*
I/II/III	61 (70.9)	15 (50.0)	

*P<0.05

TABLE II. Correlation between Zeb-1 and Clinicopathological Variables

Variables	Zeb-1 low group (n = 84), N (%)	Zeb-1 high group (n = 32), N (%)	P value
Age			
≥ 64	51 (60.7)	12 (37.5)	0.036*
≤ 63	33 (39.3)	20 (62.5)	
Gender			
Male	63 (75.0)	20 (62.5)	0.25
Female	21 (25.0)	12 (37.5)	
Tumor size			
≥ 65 mm	40 (47.6)	16 (50.0)	0.84
65 mm <	44 (52.4)	16 (50.0)	
Pathological type			
Diffuse type	44 (52.4)	24 (75.0)	0.035*
Intestinal type	40 (47.6)	8 (25.0)	
Pathological T category			
pT3/4	62 (73.8)	28 (87.5)	0.14
pT1/2	22 (26.2)	4 (12.5)	
Vessel involvement			
Yes	42 (50.0)	20 (62.5)	0.30
No	42 (50.0)	12 (37.5)	
Lymphatic vessel involvement			
Yes	70 (83.3)	29 (90.6)	0.39
No	14 (16.7)	3 (9.4)	
Lymph node metastasis			
Yes	50 (59.5)	25 (78.1)	0.083
No	34 (40.5)	7 (21.9)	
Number of lymph node metastasis			
≥ 16	8 (9.5)	9 (28.1)	0.018*
< 16	76 (90.5)	23 (71.9)	
Peritoneal metastasis			
Yes	11 (13.1)	6 (18.7)	0.56
No	73 (86.9)	26 (81.3)	
Liver metastasis			
Yes	5 (6.0)	1 (3.1)	1.0
No	79 (94.0)	31 (96.9)	
Stage			
IV	26 (31.0)	14 (43.7)	0.27
I/II/III	58 (69.0)	18 (56.3)	

P<0.05*

TABLE III. Multivariate Analysis for Predictors of Survival

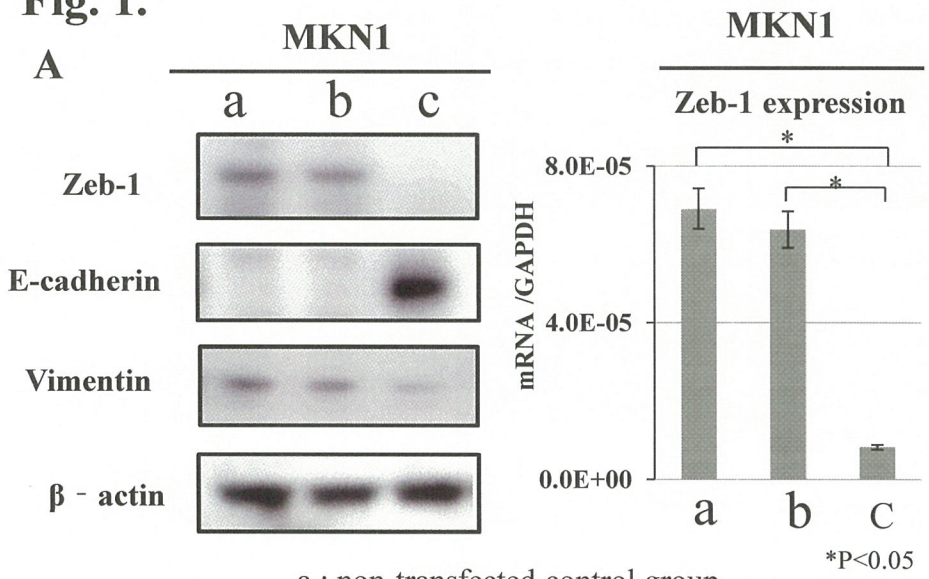
	Univariate analysis		Multivariate analysis	
Variables	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**
Age (≥64 vs. ≤63)	0.870 (0.507 - 1.50)	0.61		
Gender (male vs. female)	1.25 (0.688 - 2.44)	0.47		
Tumor size (≥65mm vs. 65mm<)	2.70 (1.55 - 4.83)	0.0004*	1.98 (1.08 – 3.70)	0.027*
Pathological type (Diffuse type vs. Intestinal type)	2.05 (1.15 - 3.84)	0.014*	1.18 (0.570 – 2.37)	0.66
Pathological T classification (pT3/4 vs. pT1/2)	11.6 (3.60 - 71.0)	<0.0001*	5.27 (1.43 -34.3)	0.0098*
Vessel involvement ((+) vs. (-))	1.83 (1.06 – 3.27)	0.0031*	1.18 (0.651 – 2.21)	0.59
Lymphatic vessel involvement ((+) vs. (-))	6.12 (1.90 – 37.4)	0.0007*	2.10 (0.489 – 14.6)	0.34
Lymph node metastasis ((+) vs. (-))	5.25 (2.52 – 12.8)	<0.0001*	1.65 (0.72 – 4.58)	0.26
Peritoneal metastasis ((+)) vs. (-))	4.92 (2.63 – 8.81)	<0.0001*	2.89 (1.46 – 5.57)	0.0028*
Liver metastasis ((+) vs. (-))	2.09 (0.630 – 5.14)	0.20		
EMT status (Mesenchymal vs. Epithelial)	2.03 (1.13 – 3.55)	0.020*	2.22 (1.13 – 4.28)	0.022*

HR: hazard ratio,

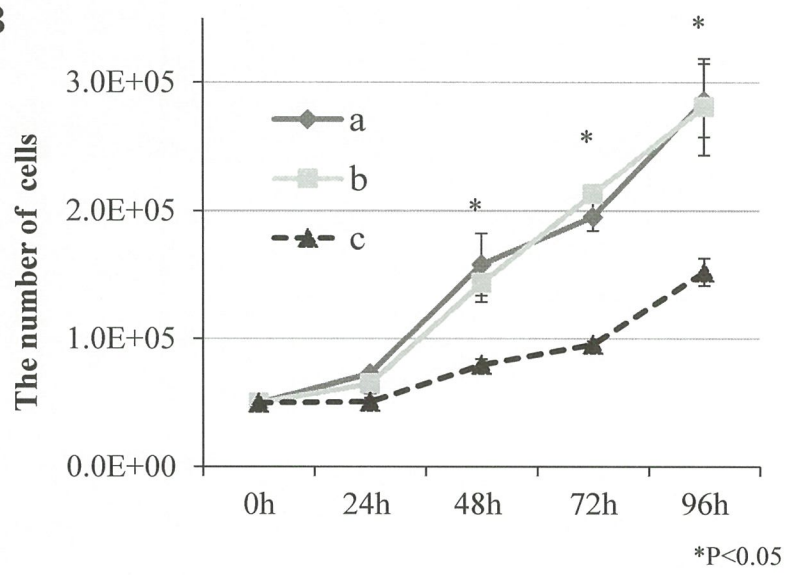
CI: confidence interval,

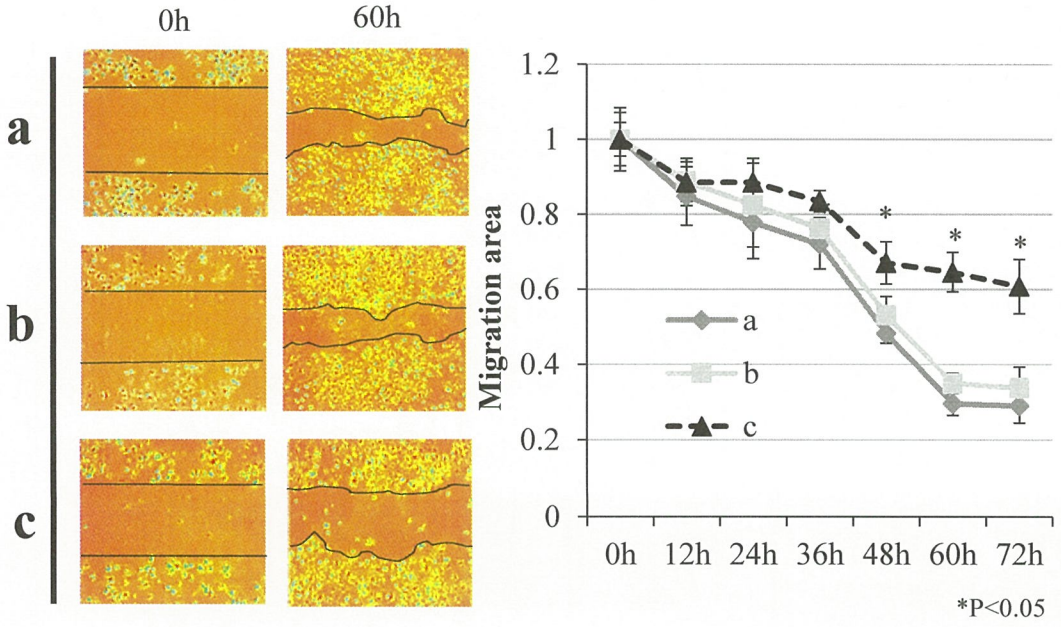
* P<0.05

Fig. 1.



a : non-transfected control group
b : control siRNA transfected group
c : Zeb-1 siRNA transfected group





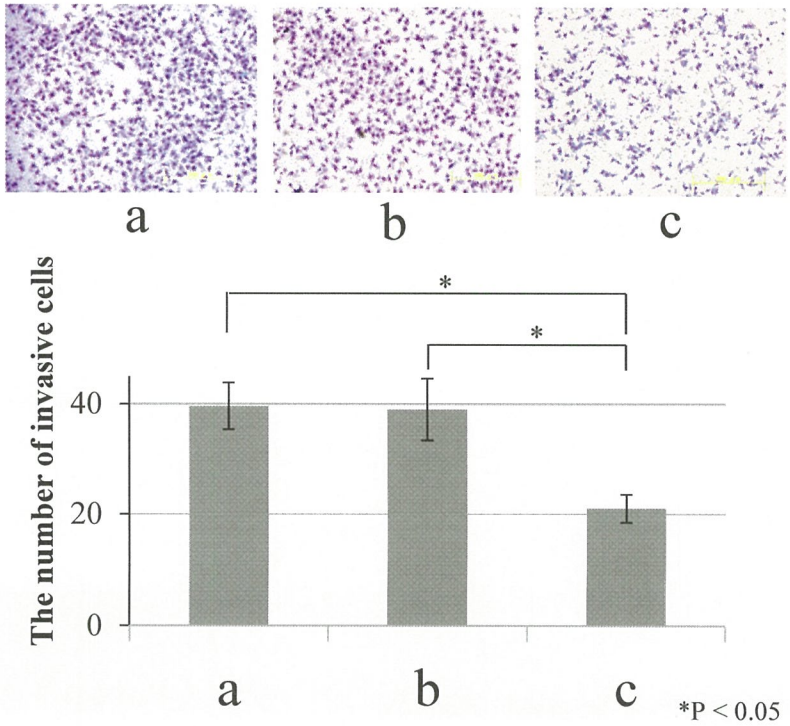
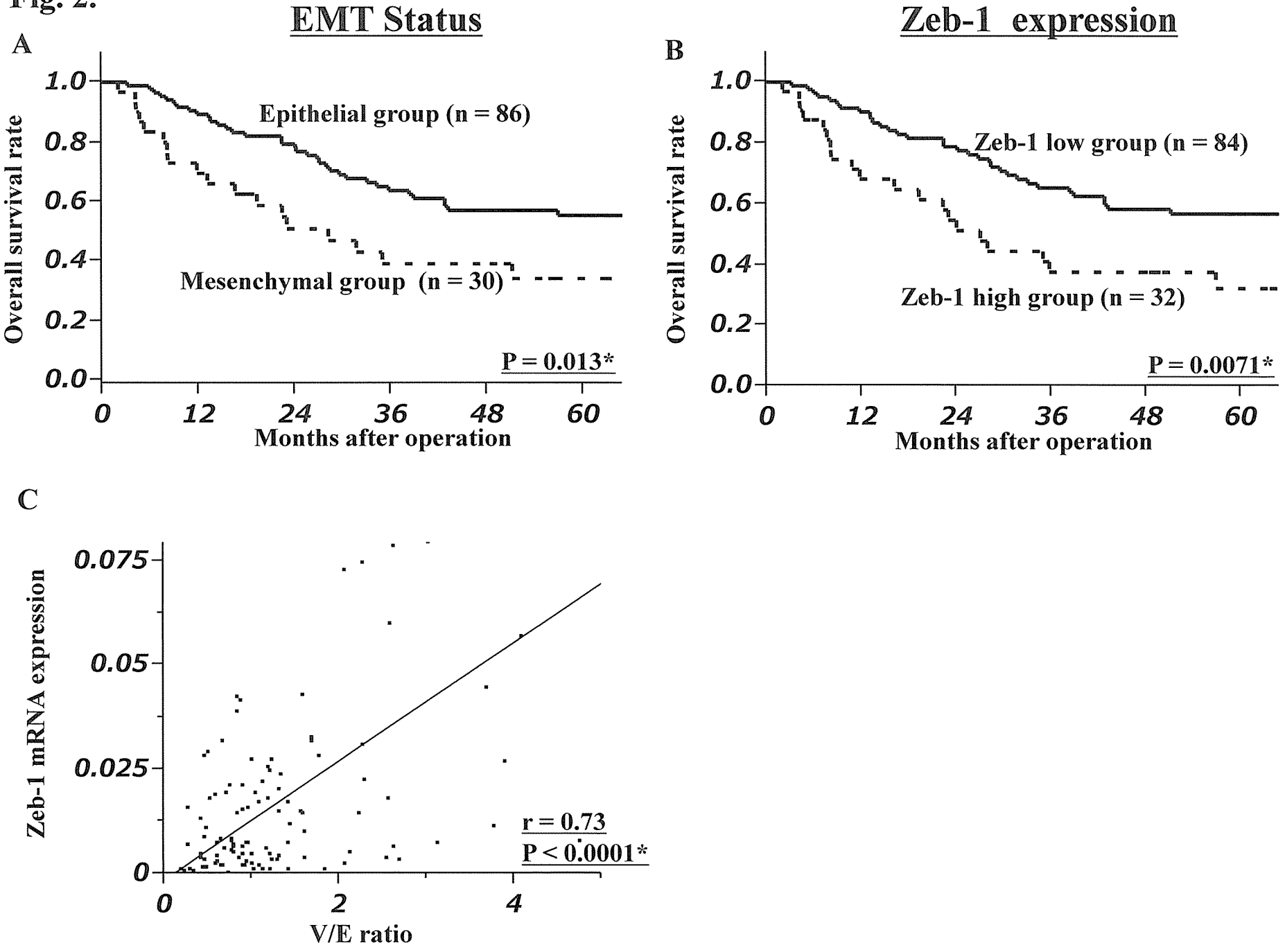


Fig. 2.


A
EMT Status
Overall survival rate
1.0
0.8
0.6
0.4
0.2
0.0
Epithelial group (n = 86)
Mesenchymal group (n = 30)
P = 0.013*
0
12
24
36
48
60
Months after operation
B
Zeb-1 expression
Overall survival rate
1.0
0.8
0.6
0.4
0.2
0.0
Zeb-1 low group (n = 84)
Zeb-1 high group (n = 32)
P = 0.0071*
0
12
24
36
48
60
Months after operation
C
Zeb-1 mRNA expression
0.075
0.05
0.025
0
r = 0.73
P < 0.0001*
0
2
4
V/E ratio