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Upregulation of integrin β4 promotes epithelial–mesenchymal transition and is a novel prognostic marker in pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDA) is a highly aggressive and often lethal malignant tumor. Several studies have shown that epithelial–mesenchymal transition (EMT) is frequently observed in clinical samples of PDA and is related to high metastatic rates and poor outcomes. To identify candidate molecules regulating EMT in PDA, we previously used cDNA microarray analysis and identified *integrin β4* (*ITGB4*) as one of the genes upregulated in high-EMT xenografts derived from PDA patients. The aim of the current study was to clarify the clinicopathological and functional significance of ITGB4 overexpression in PDA. ITGB4 upregulation in high-EMT xenografts was confirmed by immunohistochemistry. Immunohistochemical analyses of 134 surgically resected PDA cases revealed intratumoral heterogeneity with respect to ITGB4 expression and showed that cancer cells undergoing EMT often display strong diffuse ITGB4 expression. High levels of ITGB4 expression were significantly correlated with the hallmarks of EMT (solitary cell infiltration, reduced E-cadherin expression, and increased vimentin expression), with high tumor grade, and with the presence of lymph node metastasis, and showed an independent prognostic effect. Immunocytochemical analyses of PDA cell lines revealed that localization of ITGB4 changed from regions of cell–cell contact to diffuse cytoplasm and cell edges with occasional localization in filopodia during EMT. Knockdown of ITGB4 reduced the migratory and invasive ability of PDA cells. Overexpression of ITGB4 promoted cell scattering and cell motility in combination with downregulation of E-cadherin and upregulation of vimentin expression. In conclusion, we elucidated the prognostic and clinicopathological significance of ITGB4 overexpression in PDA and also the potential role for ITGB4 in the regulation of cancer invasion and EMT.
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Pancreatic cancer is the fourth most common cause of cancer death in men and women in the United States.[1] Despite medical improvements, pancreatic cancer remains one of the most lethal malignancies: the 5-year survival rate for patients with pancreatic cancer is only about 6%.[1] Pancreatic ductal adenocarcinoma (PDA), characterized by the formation of ducts resembling pancreatic ducts, is the most common histologic type of pancreatic cancer. Resectability is considered to be the most significant prognostic factor; however, at the time of diagnosis, only about 20% of patients with PDA are surgically resectable because of distant metastases or major vessel involvement.[2] Even after curative surgery, the 5-year survival rate is 10%– 25%, mainly due to the highly aggressive biological behavior of this tumor, such as the high rate of local recurrence, peritoneal dissemination, liver metastases, and lymph node recurrence.[3]

The epithelial–mesenchymal transition (EMT) is a series of cellular and molecular processes during which polarized epithelial cells lose cell–cell and cell–basement membrane interactions at the same time as acquiring mesenchymal and migratory properties. EMT is now widely accepted as an indispensable mechanism not only in embryonic morphogenesis but also in cancer progression in many organs.[4] A previous study revealed that EMT is frequently observed in clinical samples of PDA and is related to high metastatic rates and poor outcomes in patients with PDA.[5] Because the molecular networks involved in cancer EMT are complex and the essential signals seem to be different depending on the organ and cell types,[4,6] we previously conducted cluster analyses of gene expression profiles in 12 patient-derived PDA xenografts based on the frequency of EMT to identify candidate genes regulating cancer EMT in clinical PDA.[7] In

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the current study, we focused on *integrin β4* (*ITGB4*), one of the upregulated genes in high-EMT xenografts that was identified in the previous study.

The β4-integrin subunit is a transmembrane protein that heterodimerizes with the α6-integrin subunit to form a receptor for laminin. The β4-integrin subunit has an unusually long cytoplasmic domain of 1017 amino acids that has distinctive cytoskeletal and signaling functions.[8] One fundamental function of α6β4-integrin in polarized epithelial cells is to form stable attachment to the basal membrane through the formation of hemidesmosomes. In addition, ITGB4 has a potential role in signaling events associated with migration, cell growth, and survival under physiological and pathological conditions.[9–11] Prior reports indicate that ITGB4 is upregulated in multiple tumor types, including pancreatic cancer.[8] In several cancers, high levels of ITGB4 expression have been linked to poor prognosis or aggressive behavior,[12–16] but the clinicopathological significance of ITGB4 overexpression in PDA remains unclear. The aim of the current study was to clarify the clinicopathological significance of ITGB4 overexpression in PDA and to suggest the potential function of ITGB4 in cancer EMT.

MATERIALS AND METHODS

Patients and Tissue Samples

For clinicopathological analyses, we consecutively selected 134 patients with PDA who underwent pancreatectomy at Keio University Hospital (Tokyo, Japan) between 1991 and 2010. No patient received any therapy before the initial surgery. Patients with pancreatic malignancies of special types, such as intraductal papillary-mucinous neoplasia, adenosquamous carcinoma, colloid carcinoma, and undifferentiated carcinoma, were excluded from the study. The mean patient age was 65.3 years (range, 29–86 years); the male-to-female ratio was 85:49. Tumors were classified according to the World Health Organization classification or the Classification of Pancreatic Carcinoma of the Japan Pancreas Society.[17,18] Tumor stage was evaluated by the TNM staging system approved by the Union for International Cancer Control (UICC).[19] All experiments using human samples were approved by the ethics committees of the Keio University School of Medicine.

Immunohistochemistry

Immunohistochemical staining for ITGB4 was carried out on formalin-fixed, paraffin-embedded tissue sections by an immunoperoxidase method. Briefly, each section was deparaffinized, rehydrated, and incubated with 0.3% hydrogen peroxide in methanol to block the endogenous peroxidase activity. Sections were treated with 0.1% trypsin at 37 °C for 60 min, followed by blocking with 2.5% normal horse serum, and incubated with a rat monoclonal anti-β4-integrin antibody (1:100; 439-9B; eBioscience, San Diego, CA, USA). After washing in phosphate-buffered saline (PBS), the primary antibodies were visualized using an anti-rat Simple Stain Kit (Histofine Simplestain Max-PO; Nichirei, Tokyo, Japan) with 3,3′-diaminobenzidine and counterstained with hematoxylin. Staining for E-cadherin and vimentin was carried out as reported previously.[5]

Evaluation of Immunostaining for ITGB4

Expression of ITGB4 was immunohistochemically detected in peripheral nerves, normal pancreatic ducts, and endothelial cells in non-cancerous pancreatic tissues. Because the staining intensity of peripheral nerves was consistent, whereas the staining intensity in normal pancreatic ducts and endothelial cells varied depending on the size of ducts and vessels, the peripheral nerve was selected as an internal positive control in each slide. Cancer cells demonstrated three distinct staining patterns: pattern 1, no detectable or dot-like staining; pattern 2, predominant membranous (ordinarily basal/basolateral) staining with little or no cytoplasmic staining; pattern 3, equal or stronger diffuse cytoplasmic staining compared with that of peripheral nerves (Supplementary Figure 1). We considered cancer cells with staining pattern 3 to indicate ITGB4-overexpressing cells. The rate of ITGB4 overexpression was defined as the percentage of ITGB4-overexpressing cells per total cancer cells in each slide, and was carefully evaluated together by the two experienced pathologists (YM and MS). A histogram of the overexpression rate displayed a bimodal distribution (Supplementary Figure 2). The median rate was 30–40%. When the rate of ITGB4 overexpression was $>30\%$, the case was designated as having high levels of ITGB4 expression.

Evaluation of the Hallmarks of EMT in Tissue Sections

Solitary cell infiltration, reduced membranous E-cadherin expression, and increased vimentin expression were used for histological evaluation of EMT in this study. Solitary cell infiltration, defined as microscopic evidence of cancer cells singly infiltrating into the stroma, was reported to be an indicator of morphological EMT.[5] These hallmarks were evaluated as reported previously.[5]

Outcomes

Ten of the 134 patients were excluded from survival analyses because six died before hospital discharge within 30 days of surgery, three patients were lost to follow-up, and one had synchronous progressed cancer derived from intraductal papillary mucinous neoplasms. Thus, 124 patients were analyzed for survival and outcome. The final survival data were collected on 10 February 2012. The median follow-up period was 23.4 months (range, 2.4–222.6 months).

Pancreatic Cancer Cell Lines

All human pancreatic cancer cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). Conventionally, cells were cultured in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS).

Immunocytochemical Analyses

CFPAC-1 cells were incubated on culture slides (BD Biosciences, San Diego, CA, USA) with RPMI medium supplemented with 10% FBS. HPAF-II cells were cultured with RPMI medium supplemented with 10% FBS and 50 ng/ml of recombinant human EGF (R&D Systems, Minneapolis, MN, USA) for 3 days or 20 ng/ml of TGF-β (representative EMT inducer) for 4 days before fixation. Cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and probed with anti-β4-integrin rat monoclonal antibody, anti-E-cadherin (HECD-1; Alexis Biochemicals, San Diego, CA, USA) mouse monoclonal antibody, or anti-vimentin (V9; Dako, Glostrup, Denmark) mouse monoclonal antibody. The slides were rinsed with PBS, covered with FITC-conjugated anti-rat Ig (Dako) and TRITC-conjugated anti-mouse Ig (Dako), and visualized using an LSM 510 Meta confocal microscope (Carl Zeiss, Overkochen, Germany). To visualize F-actin, we used rhodamine-phalloidin (Invitrogen).

For the detection of EMT-related transcriptional factors, anti-SNAI1 rabbit polyclonal antibody (Abcam, Cambridge, UK), anti-SNAI2 rabbit polyclonal antibody (Abcam), anti-ZEB1 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-ZEB2 mouse monoclonal antibody (Santa Cruz Biotechnology) were used as primary antibodies. After washing in PBS, the slides were incubated with Alexa Fluor 488 goat anti-rabbit Ig (Life Technologies, CA, USA) for SNAI1 and SNAI2, with Alexa Fluor 488 goat anti-mouse Ig (Life Technologies) for ZEB2, or with FITC-conjugated chicken anti-goat Ig (Santa Cruz Biotechnology) for ZEB1.

Western blot Analyses

Western blot analyses were performed as described previously.[20] For the extraction of phosphoprotein, we used modified RIPA buffer: 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF and protease inhibitors.

Semiquantitative RT-PCR Analyses

RT-PCR analyses were performed as described previously.[21] Primer sequences were shown in Supplementary Table 1.

RNA Interference

RNA interference was performed as described previously.[20] The target sequences for knockdown of ITGB4 were as follows: siITGB4a, 5′-GTCGCTGATGTGATAACCT-3′ and siITGB4b, 5′-GCTACTGTTGGCTGGATAA-3′. As a control, negative control small interfering RNA (siRNA) was purchased from Qiagen.

Migration and Invasion Assays

Specific numbers of cells (3×10^4 for CFPAC-1 and 1.8×10^5 for AsPC-1) were dispersed to each of three independent upper chambers of BD Falcon Cell Culture Inserts (24-well plates, pore size 8 μm; BD Biosciences). For the invasion assay, the upper chambers were precoated with 10 $\mu g/cm^2$ BD Matrigel Basement Membrane Matrix (BD Biosciences) by a thin coating method according to the manufacturer's instructions. Cells migrating to the lower surface of the membrane after 24 h of incubation were stained with Diff-Quik (Kokusai Shiyaku, Kobe, Japan). The percentage migration/invasion was defined as the ratio of the mean number of migrated/invaded cells to that of control cells.

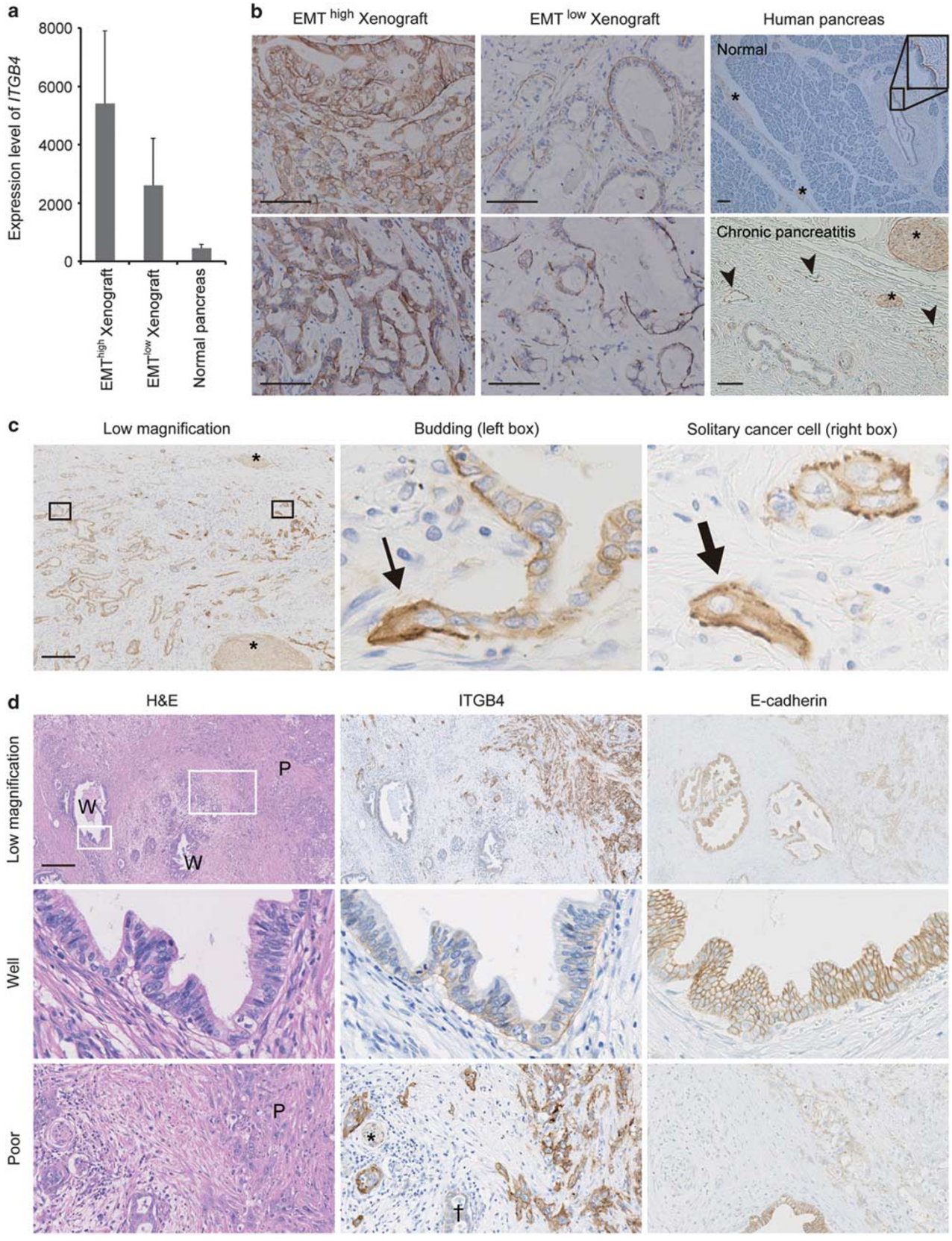
Transfection

Human β4 *integrin* full-coding cDNA was amplified by reverse transcription-PCR and inserted into pcDNA 3.1 (+) (Invitrogen). CFPAC-1 cells were transfected with the plasmid. Lipofectamine LTX (Invitrogen) and stable transfectants were selected with G418 (Invitrogen), according to the manufacturer's instructions.

Statistical Analyses

Data were analyzed using the IBM SPSS statistics package (SPSS, Chicago, IL, USA), version 21. Correlation analyses on several clinicopathologic factors were tested with the χ^2 test or the Fischer's exact test. Survival curves were calculated from the date of surgery using the Kaplan–Meier method and were compared using the log-rank test. Univariate and multivariate analyses were examined using the Cox proportional hazards regression model. Statistical differences for migration and invasion assays were performed using the two-sided, unpaired *t*-test. *P*-values <0.05 were considered statistically significant in all analyses.

Figure 1 Overexpression of integrin β4 (ITGB4) is associated with epithelial–mesenchymal transition (EMT) in xenografts and clinical tissues of pancreatic ductal adenocarcinoma (PDA). (**a**) The average gene expression levels of *ITGB4* in patient-derived PDA high-EMT xenografts (EMT^{high} xenograft), in those with low-EMT (EMT^{low} xenograft), and in normal human pancreatic tissues. (**b**) Immunostaining for ITGB4 in xenografts and human non-cancerous pancreas. ITGB4 expression was detected in peripheral nerves (asterisks), pancreatic ducts, and endothelial cells (arrowheads) in both normal human pancreas and chronic pancreatitis. In normal pancreatic ducts, ITGB4 shows basal localization (inset). (**c**) Immunostaining for ITGB4 in surgically resected PDA tissues. Peripheral nerves (asterisks) serve as internal positive controls. The low magnification image shows that the staining intensity of ITGB4 is stronger at the invasive front (right) than in the tumor center (lower left). At high magnification, a budding cell (thin arrow) and a scattering cancer cell (thick arrow) display strong diffuse expressions of ITGB4. (**d**) Low magnification image reveals that the staining intensity of ITGB4 is stronger in poorly differentiated components (P) than in well-differentiated glands (W). Weak predominantly basal expression of ITGB4 is shown in cancer cells of well-differentiated glands, which demonstrate preserved membranous E-cadherin expression, whereas strong diffuse ITGB4 expression is observed in poorly differentiated cancer cells, which show reduced E-cadherin expression. Note that the intensity of ITGB4 expression in poorly differentiated components is higher than in peripheral nerves (asterisk) and in carcinoma cells forming defined glands (dagger). Scale bars: 100 μm (**b**) and 200 μm (**c** and **d**).


a
Expression level of ITGB4
8000
6000
4000
2000
0
EMT high Xenograft
EMT low Xenograft
Normal pancreas
b
EMT high Xenograft
EMT low Xenograft
Human pancreas
Normal
Chronic pancreatitis
c
Low magnification
Budding (left box)
Solitary cancer cell (right box)
d
H&E
ITGB4
E-cadherin
Low magnification
Well
Poor
P
W
W
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RESULTS

ITGB4 is Upregulated in Patient-Derived High-EMT PDA Xenografts

According to a previous study analyzing gene expression profiles in 12 patient-derived PDA xenografts,[7] expression levels of *ITGB4* were higher in all xenografts than those in normal human pancreatic tissues (Figure 1a). The average level of *ITGB4* expression in high-EMT PDA xenografts was more than two times that of low-EMT PDA xenografts (Figure 1a). ITGB4 upregulation in high-EMT xenografts was confirmed by immunohistochemistry: diffuse strong ITGB4 expression (staining pattern 3) was characteristic in high-EMT xenografts, whereas predominant basal expression (staining pattern 2) was evident in low-EMT xenografts (Figure 1b). In non-cancerous human pancreas, ITGB4 was immunohistochemically detected only in pancreatic ducts, peripheral nerves, and endothelial cells, as mentioned above (Figure 1b).

Overexpression of ITGB4 is Correlated With Hallmarks of EMT in Clinical PDA

To validate the association between expression levels of ITGB4 and the frequency of EMT in clinical PDA, we conducted immunohistochemical analyses for ITGB4 in 134 surgically resected PDA samples. Immunostaining for ITGB4 was heterogeneous in cancer tissue: cancer cells displaying well-defined glandular structures tended to have predominant basal/basolateral membranous staining for ITGB4 (pattern 2), whereas poorly differentiated carcinoma components, including budding cells and solitary infiltrating cancer cells at invasive fronts, frequently presented diffuse cytoplasmic and strong ITGB4 expression (pattern 3; Figures 1c and d). Cancer cells with staining pattern 3 often exhibited strong staining intensity also in the cell membrane or cell edge (Figure 1c). Reduced membranous E-cadherin expression, which is a representative marker of EMT, was mostly observed in ITGB4-overexpressing cells (Figure 1d). High levels of ITGB4 expression correlated significantly with the histological hallmarks of EMT, that is, a high degree of solitary cell infiltration ($P<0.001$), reduced E-cadherin expression ($P<0.001$), and increased vimentin expression ($P<0.001$; Table 1).

Table 1 Correlation between ITGB4 expression and the hallmarks of EMT

Factors	Total, *n* (%)	ITGB4 expression, *n* (%)		*P*-value
		Low	High	
Solitary cell infiltration				<0.001[a]
Low degree	37 (28)	27 (20)	10 (8)	
High degree	97 (72)	36 (27)	61 (46)	
E-cadherin expression				<0.001[a]
Preserved	56 (42)	48 (36)	8 (6)	
Reduced	78 (58)	15 (11)	63 (47)	
Vimentin expression				<0.001[a]
Non-increased	80 (60)	51 (38)	29 (22)	
Increased	54 (40)	12 (9)	42 (31)	

[a]Significant.

Clinicopathological Significance of ITGB4 Overexpression in PDA

Table 2 summarizes the demographic and clinicopathological factors of the 134 PDAs categorized by expression levels of ITGB4. High levels of ITGB4 expression significantly correlated with high tumor grade and the presence of lymph node metastasis. No statistical correlation was found with age, gender, tumor size, location, major vessel involvement, or margin status.

To clarify the prognostic value of ITGB4 overexpression in pancreatic cancers, we examined overall survival analyses. The Kaplan–Meier curves indicated that the group with high levels of ITGB4 expression had a significantly worse outcome than the group with low ITGB4 expression ($P<0.001$; Figure 2a). The results of univariate and multivariate analyses on the effect of clinicopathological factors are summarized in Table 3. Univariate analyses showed that tumor size, grade, margin status, lymph node metastasis, and levels of ITGB4 expression significantly correlated with overall survival. Variables with a *P*-value <0.10 in the univariate analyses were used in the multivariate model. Multivariate analyses revealed the independent prognostic effect of the grade, major vessel involvement, and levels of ITGB4 expression.

To examine whether ITGB4 expression has additional prognostic information beyond that of tumor grade alone, we further performed combined survival analyses for tumor grade and ITGB4 expression. We found that patients with grade 1 and 2 tumors with low levels of ITGB4 expression had significantly better overall survival than those in the other three risk groups: those with grade 3 tumors with high levels of ITGB4 expression ($P<0.001$), grade 3 tumors with low levels of ITGB4 expression ($P=0.009$), and grade 1 and 2 tumors with high levels of ITGB4 expression ($P<0.001$; Figure 2b). There was no significant difference in prognosis between those with grade 1 and 2 tumors with high levels of ITGB4 expression and those with grade 3 tumors.

ITGB4 Distribution Changes During EMT in PDA cells

We further examined PDA cell lines to analyze the direct link between ITGB4 and EMT. To observe the subcellular localization of endogenous ITGB4, we performed immunocytochemical analyses in CFPAC-1 and HPAF-II cells. Both cell types exhibited two differential distribution patterns with respect to ITGB4 expression: a mainly membranous distribution along with cell–cell contact, and diffuse cytoplasmic distribution (Figures 3a and b). While cancer cells with

Table 2 Correlation between ITGB4 expression and clinicopathological factors

Factors	Total, *n* (%)	ITGB4 expression, *n* (%)		*P*-value
		Low	High	
Age (years)				0.072
<60	33 (25)	20 (15)	13 (10)	
≥60	101 (75)	43 (32)	58 (43)	
Gender				0.147
Male	85 (63)	44 (33)	41 (31)	
Female	49 (37)	19 (14)	30 (22)	
Tumor size				0.327
<3 cm	77 (57)	39 (29)	38 (28)	
≥3 cm	57 (43)	24 (18)	33 (25)	
Tumor location				0.499
Pancreas head	89 (66)	40 (30)	49 (37)	
Body and tail	45 (37)	23 (17)	22 (16)	
Grade				< 0.001[b]
1/2	100 (75)	58 (43)	42 (31)	
3	34 (25)	5 (4)	29 (22)	
Lymphatic invasion				1.000[a]
Absent	4 (3)	2 (1)	2 (1)	
Present	130 (97)	61 (46)	69 (52)	
Neural invasion				0.051[a]
Absent	7 (5)	6 (4)	1 (1)	
Present	127 (95)	57 (43)	70 (52)	
Major vessel involvement				0.464
Negative	85 (63)	42 (31)	43 (32)	
Positive	49 (37)	21 (16)	28 (21)	
Margin status				0.815
Negative	97 (72)	45 (34)	52 (39)	
Positive	37 (28)	18 (13)	19 (14)	
Lymph node metastasis				0.022[b]
Negative	27 (20)	18 (13)	9 (7)	
Positive	107 (80)	45 (34)	62 (46)	
UICC stage				NA
IA	3 (2)	1 (1)	2 (1)	
IB	2 (1)	1 (1)	1 (1)	
IIA	22 (16)	16 (12)	6 (4)	
IIB	104 (78)	44 (33)	60 (45)	
III	2 (1)	1 (1)	1 (1)	
IV	1 (1)	0 (0)	1 (1)	

Abbreviations: NA, not assessed; UICC, Union for International Cancer Control.
[a]Analyzed by the Fischer's exact test; others were analyzed by the χ^2 test.
[b]Significant.

membranous ITGB4 distribution showed a cohesive phenotype, those with diffuse ITGB4 distribution were mainly observed at the periphery of cohesive cancer nests or were scattered singly. Cancer cells with diffuse cytoplasmic ITGB4 expression frequently expressed cytoplasmic vimentin (Figure 3a) and often exhibited loss of linear membranous expression of E-cadherin (Figure 3b). Some cancer cells with diffuse distribution of ITGB4 displayed accumulation of ITGB4 at cellular leading edges (Figure 3b, bottom focus).

Further studies were performed to investigate the association of ITGB4 with the actin cytoskeleton at the cellular leading edge. Confocal fluorescence microscopic analyses revealed that ITGB4 was localized at the tip or shaft of some of filopodia, whereas ITGB4 was not detected at the leading edge of lamellipodia (Figure 3c and Supplementary Figure 3). These data indicated that localization of ITGB4 changed from regions of cell–cell contact to diffuse cytoplasm, frequently concurrent with subcellular localization at leading edges and filopodia during EMT in PDA cell lines.

Knockdown of ITGB4 Decreases Cell Motility in PDA Cells

To assess the function of ITGB4 in PDA cells, knockdown of ITGB4 in CFPAC-1 and AsPC-1 cells, which expressed ITGB4 at a relatively high level (Supplementary Figure 4), was performed using two siRNA molecules, designated siITGB4a and siITGB4b. Figure 4a shows the results of western blot analyses that confirmed knockdown of ITGB4 by both siRNA molecules. There were no significant differences in the expression of focal adhesion kinase (FAK) and phospho-FAK by knockdown of ITGB4 (Supplementary Figure 5). Cell scattering was notably suppressed in CFPAC-1 transfectants by siITGB4a and siITGB4b compared with non-silenced cells (Supplementary Figure 6). Migration and invasion assays demonstrated that cell motility was significantly suppressed by knockdown of ITGB4 in both CFPAC-1 and AsPC-1 transfectants (Figure 4b).

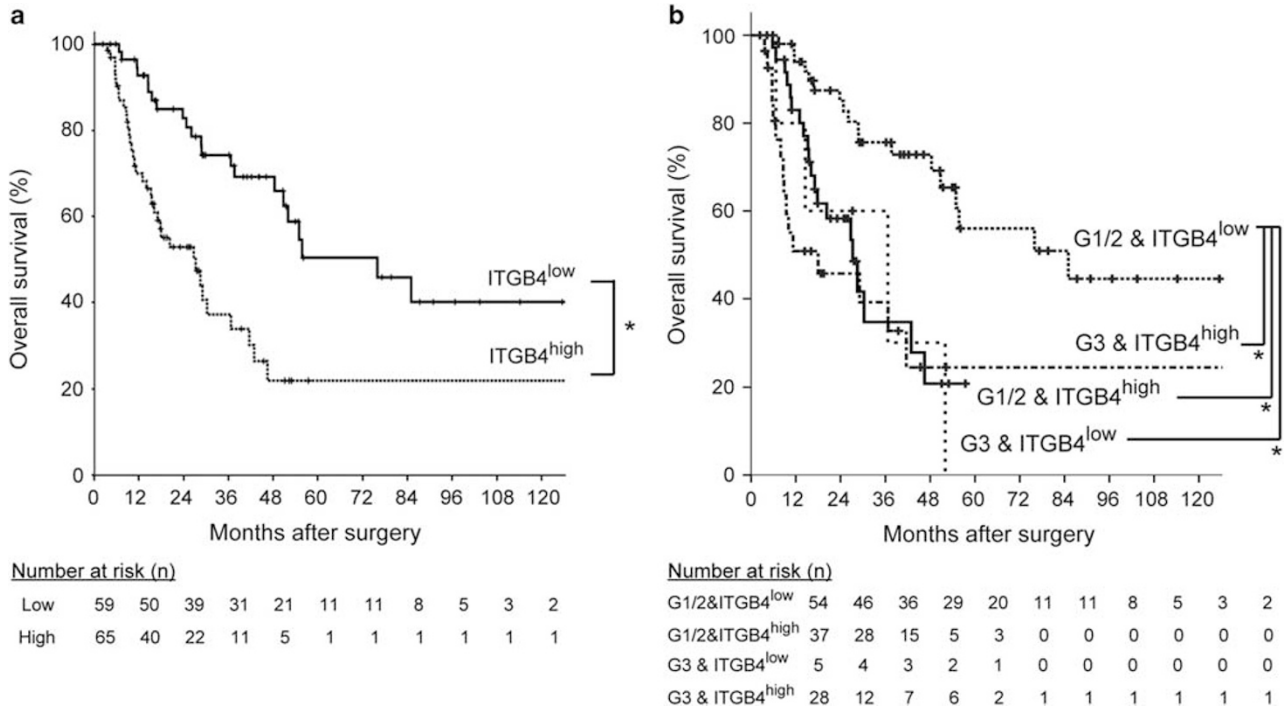

Figure 2 Survival analyses for overall survival. (**a**) Overexpression of *integrin β4* (ITGB4) correlates with poor prognosis in terms of overall survival. (**b**) Combined survival analyses of the tumor grade and the levels of ITGB4 expression. The numbers at risk for each group are listed at the bottom of the each figure.

Overexpression of ITGB4 Promotes Cell Motility and EMT in PDA Cells

To confirm the role of ITGB4 in cell motility, CFPAC-1 single-cell clones that stably overexpress ITGB4 were established and designated CF/FL-1 and CF/FL-2. CFPAC-1 single-cell clones transfected with empty vector (designated as vector-1 and vector-2) and parental CFPAC-1 cells were used as controls. Expression levels of ITGB4, E-cadherin, vimentin, FAK, and phospho-FAK were evaluated by western blot analyses (Figure 4c and Supplementary Figure 7). Both CF/FL-1 and CF/FL-2 cells were confirmed to have increased expression of ITGB4 and vimentin in protein levels. E-cadherin expression was reduced in CF/FL-1 and CF/FL-2 cells. CF/FL-2 cells, which had slightly higher ITGB4 expression levels than CF/FL-1 cells, exhibited enhanced reduction in E-cadherin expression compared with CF/FL-1 cells. FAK expression was slightly increased by ITGB4 overexpression, but there were no significant difference for phospho-FAK expression. Panc-1 cells that stably overexpress ITGB4 were also established, and these cells showed appreciably reduced E-cadherin expression compared with control cells in western blot analyses (Supplementary Figure 8). Examination using phase-contrast microscopy revealed that cell scattering was considerably promoted in CF/FL-1 and CF/FL-2 cells compared with control cells (Figure 4d). Scratch assays revealed that cell spreading into the wound space was enhanced in CF/FL-2 cells compared with control cells (Supplementary Figure 9). The functions of ITGB4 were further evaluated by migration and invasion assays using transwells. Both migrated and invaded cell numbers were significantly increased in CF/FL-1 and CF/FL-2 cells compared with control cells (Figure 4e). Migrated and invaded cell numbers increased stepwise with the expression level of ITGB4. Considering that cell proliferation was not affected by transfection of ITGB4 (data not shown), the increased number of migrated and invaded cells was considered to be a result of increased cell motility due to overexpression of ITGB4.

To investigate the association of ITGB4 overexpression with EMT-related transcription factors, we further performed RT-PCR analyses (Figure 4f). ZEB1 expression was increased in both CF/FL-1 and CF/FL2 cells. SNAI1 (Snail) expression was increased in CF/FL-2 cell. ZEB2 exhibited slightly increased expression only in CF/FL-1 cells. Expression levels of SNAI2 (Slug) and E47/E12 demonstrated no significant difference between ITGB4-overexpressing cells and control cells. TWIST expression was not detected by RT-PCR analyses (data not shown). Immunocytochemical analyses were performed to validate the expression of SNAI1, SNAI2, ZEB1, and ZEB2 at protein levels (Supplementary Figure 10). SNAI1, ZEB1, and ZEB2 expression were observed especially in the nuclei of scattering cells, which also expressed vimentin, whereas SNAI2 expression was not observed in the nuclei of CFPAC-1 transfectants. The nuclear positivities of SNAI1, ZEB1, and ZEB2 expression were considerably higher in ITGB4-overexpressing cells compared with control cells.

Table 3 Univariate and multivariate analyses of factors associated with overall survival

Factors	Univariate		Multivariate	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (years)				
<60 *vs* ≥ 60	1.316 (0.726–2.387)	0.366		NA
Gender				
Male *vs* female	0.668 (0.375–6.191)	0.171		NA
Tumor size (cm)				
<3 *vs* ≥ 3	2.347 (1.384–3.980)	0.002	1.646 (0.943–2.872)	NS
Tumor location				
Pancreas head *vs* body or tail	0.549 (0.300–1.002)	0.051	0.533 (0.277–1.024)	NS
Grade				
1/2 *vs* 3	2.496 (1.445–4.311)	0.001	2.267 (1.224–4.200)	0.009
Lymphatic invasion				
Absent *vs* present	1.508 (0.208–10.923)	0.685		NA
Neural invasion				
Absent *vs* present	4.809 (1.662–34.907)	0.120		NA
Major vessel involvement				
Absent *vs* present	1.659 (0.986–2.796)	0.057	1.848 (1.016–3.364)	0.044
Margin status				
Negative *vs* positive	1.782 (1.067–3.125)	0.044	1.363 (0.741–2.507)	NS
Lymph node metastasis				
Negative *vs* positive	2.403 (1.175–4.912)	0.016	1.355 (0.619–2.966)	NS
ITGB4 expression level				
Low *vs* high	2.928 (1.685–5.089)	<0.001	2.218 (1.211–4.060)	0.010

Abbreviations: CI, confidence interval; HR, hazard ratio; NA, not assessed; NS, not significant.

DISCUSSION

Our findings reveal the clinicopathological significance of ITGB4 overexpression in PDA and also suggest a potential role for ITGB4 in the regulation of cancer invasion and EMT in PDA cells.

High levels of ITGB4 expression have shown significant correlations with high tumor grade and the presence of lymph node metastases and have also demonstrated an independent prognostic effect. Using cDNA microarray analyses, Nakamura *et al*[22] found that *ITGB4* is one of the upregulated genes associated with lymph node metastasis in pancreatic cancer. Recently, Crus-Monserrate *et al*[23] reported that ITGB4 expression is markedly upregulated during the multistep carcinogenesis of PDA. However, the clinicopathological significance of ITGB4 overexpression in pancreatic cancer remained unclear. We believe that our study is the first

to describe the prognostic significance of ITGB4 overexpression in PDA. The increased expression of ITGB4 is also known to be linked to poor prognosis or aggressive behavior in several other cancers, including malignancies of the head and neck, breast, bladder, and thyroid gland.[12–16] Although our study was retrospective and included surgically resectable cases, our findings suggested that ITGB4 could be a novel molecular marker predicting the aggressiveness and outcome of pancreatic cancers. In addition, combined survival analyses for tumor grade and ITGB4 expression suggested that ITGB4 can provide additional prognostic information beyond that of tumor grade, which is routinely reported in pathology reports.

Immunohistochemical analyses revealed intratumoral heterogeneity with respect to ITGB4 expression in PDA tissue. Recently, it has become clear that PDA exhibits not

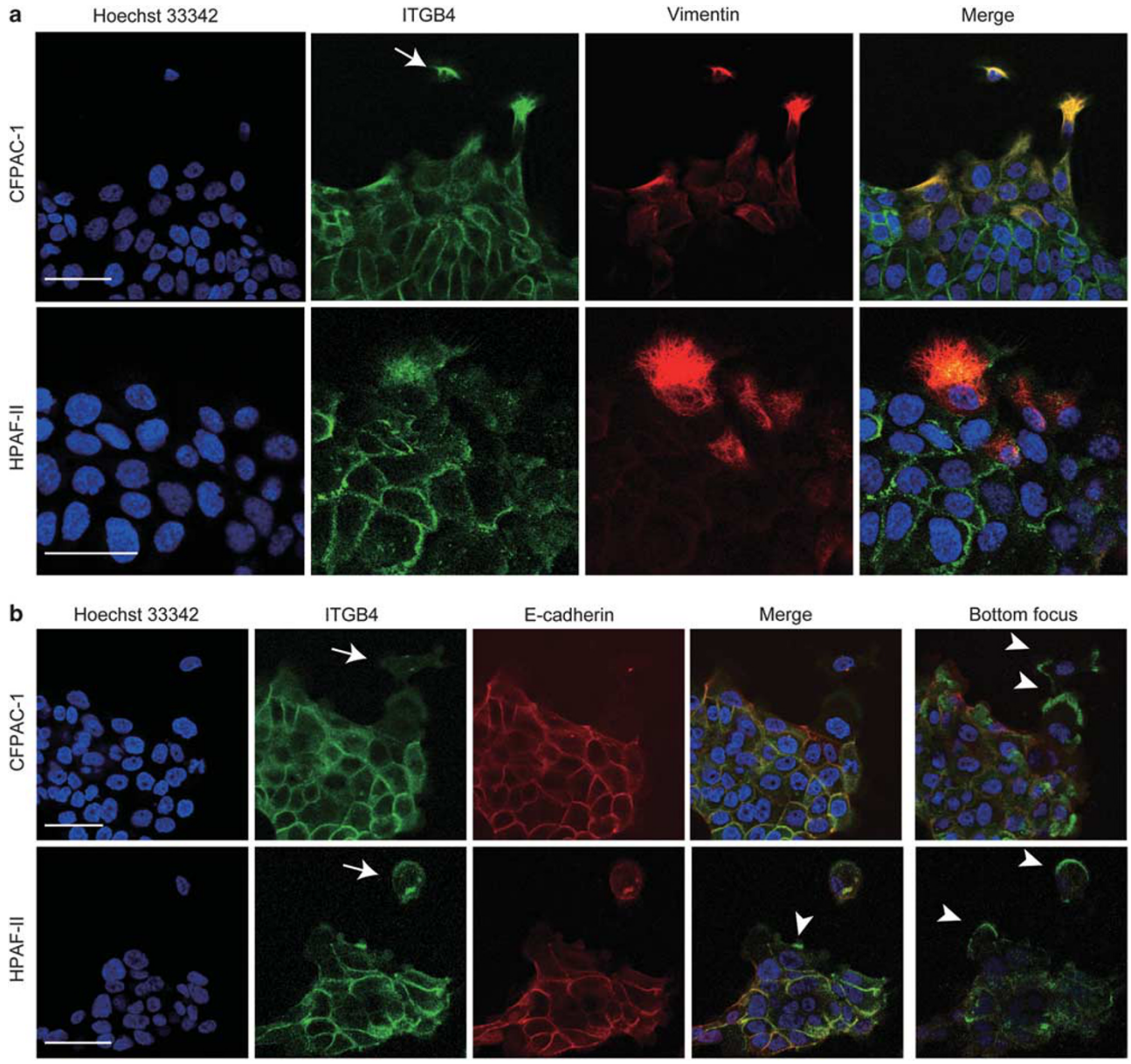

Figure 3 *integrin β4* (ITGB4) distribution changes during epithelial–mesenchymal transition (EMT) in pancreatic ductal adenocarcinoma (PDA) cells. (**a**) Immunocytochemical analyses present subcellular localization of ITGB4 (green) and vimentin (red) in CFPAC-1 and HPAF-II cells. Cancer cells with diffuse cytoplasmic localization of ITGB4 concurrently expressed vimentin, whereas no vimentin expression was detected in those with mainly membranous localization of ITGB4 along with cell–cell contact. (**b**) Immunocytochemistry in CFPAC-1 and HPAF-II cells probed by anti-ITGB4 antibody (green), anti-E-cadherin antibody (red) and Hoechst33342 (blue). Merged images at bottom focus revealed the accumulation of ITGB4 at leading edges (white arrowheads). White arrows in (**a** and **b**) indicate singly scattering cells. (**c**) In cellular leading edges, ITGB4 localizes at the filopodia (yellow arrows) but not at the edge of lamellipodium (L) in CFPAC-1 and HPAF-II cells. Scale bars: 50 μm (**a** and **b**) and 25 μm (**c**).

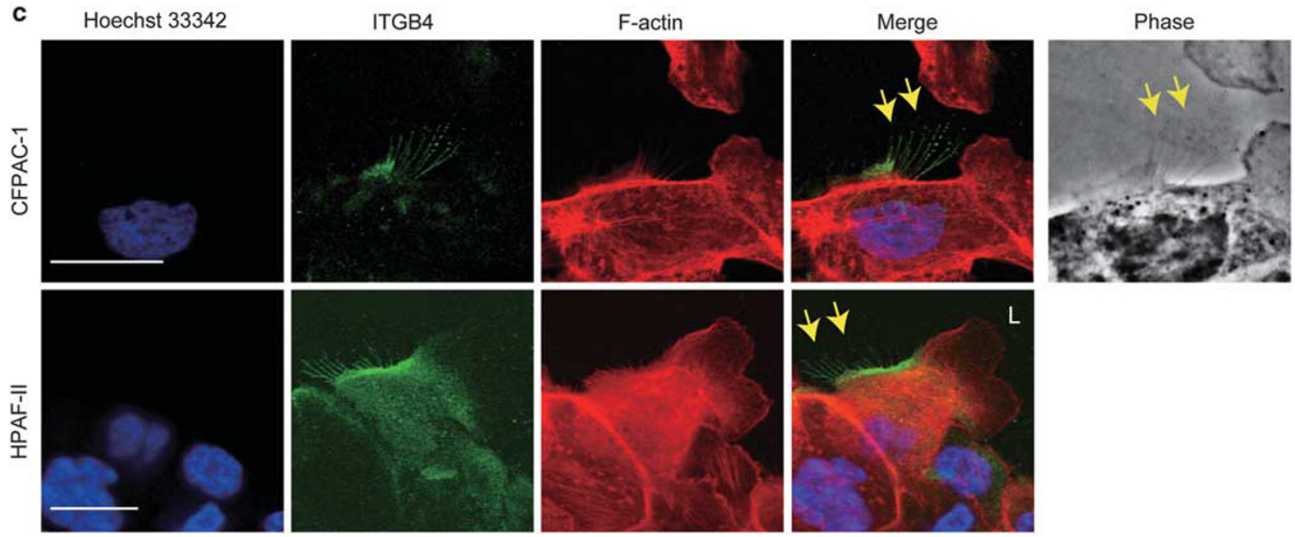

Figure 3 Continued.

only morphological but also genomic intratumoral heterogeneity.[24,25] We found that poorly differentiated adenocarcinoma components, including budding cells and solitary infiltrating cancer cells at invasive fronts, frequently display strong diffuse ITGB4 expression. Our findings are compatible with the prior study reporting that ITGB4 expression was somewhat heterogeneous and that abundant expression of ITGB4 was seen in association with invasive fronts.[26] Moreover, our results revealed that patients with grade 1 and 2 tumors with high levels of ITGB4 expression had significantly worse overall survival than those with grade 1 and 2 tumors with low levels of ITGB4 expression. These findings strongly suggest that ITGB4 is upregulated in highly invasive cancer components and that ITGB4 could be a potential biomarker indicating aggressive cancer cells in PDA tissues.

Our results have also shown that diffuse cytoplasmic distribution of ITGB4 expression, frequently concurrent with subcellular localization at leading edges, is correlated with an invasive mesenchymal phenotype both in clinical samples and in PDA cell lines. Underwood *et al*[27] showed by electron microscopic analyses that increased concentrations of ITGB4 are found in cytoplasmic vesicles within migrating keratinocytes and proposed that cytoplasmic localization of ITGB4 is caused by increased integrin trafficking. These findings lead us to surmise that cytoplasmic localization of ITGB4 represents increased intracellular trafficking of newly formed ITGB4 and/or translocated ITGB4 from the basolateral membrane to the leading edge. On the other hand, our results agree with those reported by Rabinovitz *et al*[28] showing that $\alpha 6\beta 4$-integrin functions in carcinoma migration through the formation and stabilization of filopodia. Considering that invading PDA cells are known to synthesize and deposit laminin-5,[29] which is the main ligand of $\alpha 6\beta 4$ integrin, leading to migration on the newly deposited laminin-5, we suggest that ITGB4 accumulated at leading edges and in filopodia by increased integrin trafficking is involved in adhesion to newly deposited laminin to promote leading-edge protrusion and cell migration.

We have shown that overexpression of ITGB4, which was initially identified as one of the EMT signature genes by cDNA microarray analyses,[7] correlates with the hallmarks of EMT in clinical PDA tissues and promotes cell motility in combination with promotion of the EMT phenotype *in vitro*. In addition, we confirmed that knockdown of ITGB4 significantly suppresses cancer cell motility. Our results indicated that ITGB4 overexpression promotes EMT phenotypes. In our study, phosphorylation of FAK was not changed by knockdown or overexpression of ITGB4 in pancreatic cancer cells. These findings suggest that FAK signaling is not involved in the regulation of cancer EMT by ITGB4 overexpression. On the other hand, a number of reports have revealed that ITGB4 combines with several oncogenic receptor tyrosine kinases, including c-Met, ErbB1, and ErbB2, to amplify the signaling pathways that accelerate cancer invasion.[30–32] Because these growth factor receptors are overexpressed in a large number of patients with pancreatic cancers and are involved in cancer progression,[33,34] upregulated ITGB4 may associate with some of these overactive receptor tyrosine kinases, such as EGFR and c-Met, which are well-known EMT inducers, thereby promoting tumor invasion and EMT in pancreatic cancer.

We have shown that expression levels of SNAI1, ZEB1, and ZEB2, which are well-known potent EMT-related transcription factors, were increased by the overexpression of ITGB4 in CFPAC-1 cells. Immunocytochemical analyses indicated that these transcriptional factors were accumulated in the nuclei of scattering cancer cells undergoing EMT. These results lead us to surmise that overexpression of ITGB4 promotes EMT via the upregulation of several EMT-related

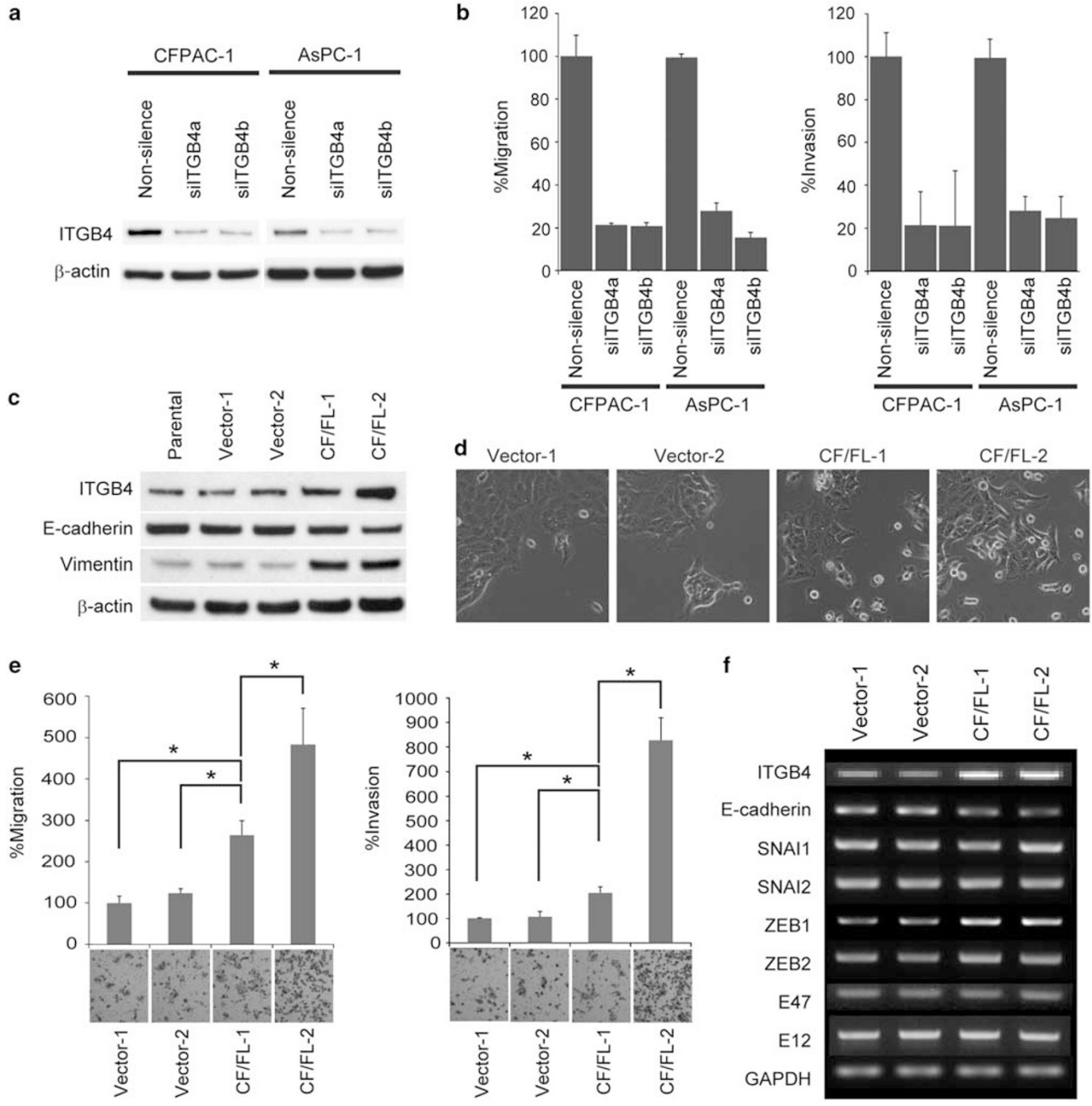

Figure 4 Overexpression of integrin $\beta 4$ (ITGB4) promotes cell motility and epithelial–mesenchymal transition (EMT) in pancreatic cancer cells. (**a**) Western blot analyses using lysates of CFPAC-1 and AsPC-1 cells treated with small interfering (si)ITGB4a, siITGB4b and negative control small interfering RNA (siRNA). (**b**) Migration and invasion assays showed significantly reduced cell migration and invasion in CFPAC-1 and AsPC-1 cells by knockdown of ITGB4. (**c**) Western blot analyses using lysates of CFPAC-1 clones stably overexpressing ITGB4 (CF/FL-1 and CF/FL-2), control cells transfected with empty vectors (vector-1 and vector-2), and parental CFPAC-1 cells. (**d**) Cell scattering was noted in CFPAC-1 cells with ITGB4 overexpression compared with control cells. (**e**) Numbers of migrated and invaded cells increased stepwise with the expression level of ITGB4. Asterisks indicate significance by the *t*-test ($P<0.05$). (**f**) Semiquantitative reverse transcription-PCR (RT-PCR) analyses for EMT-related transcriptional factors in CFPAC-1 transfectants.

transcriptional factors. Some reports reveal that more than half of patients with pancreatic cancers display expression of SNAI1/2 and ZEB1/2.[35,36] This study might indicate one of the possible mechanisms regulating transcriptional factors like SNAI1 in pancreatic cancers. However, the molecular networks regulating EMT-related transcriptional factors are extremely complex and seem to be quite different from patients to patients.[4] Further studies are needed to determine how ITGB4 is involved in the upregulation of EMT-related transcriptional factors.

In conclusion, this is the first report addressing the prognostic significance of ITGB4 overexpression in pancreatic cancer. Our findings indicate that ITGB4 may have an essential role in the regulation of cancer invasion and EMT. Considering our results and prior reports discussing the function of ITGB4 in cancer cells,[8] ITGB4 might be an attractive therapeutic target because disruption of β4 signaling leads to suppressed cancer progression. The precise molecular mechanism of ITGB4's role in the progression of pancreatic cancer remains to be clarified, but our findings suggest that ITGB4 might be a novel biomarker for predicting tumor aggressiveness for this highly malignant cancer.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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