SPANDIDOS PUBLICATIONS

Targeting ß1 integrin restores sensitivity to docetaxel of esophageal squamous cell carcinoma

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Abstract. Esophageal squamous cell carcinoma (ESCC) is a common and highly fatal cancer in Japan. Systemic chemotherapy is used, but some tumors show resistance to it. The mechanisms of tumor resistance to chemotherapy remain largely unknown. We determined the chemosensitivity of 15 ESCC cell lines (TE-1-5, TE-8-15, KYSE140 and KYSE150) to docetaxel by clonogenic and MTT assays. We used cDNA microarray analysis and quantitative RT-PCR to determine which genes might determine resistance to docetaxel. Small interfering RNA (siRNA) was used to suppress gene expression and its effect on the chemosensitivity of the cell was determined. The cell line with the most resistance to docetaxel was TE-2. Using microarray analysis, we identified *ß1 integrin (ITGB1)* to be overexpressed in this cell line. Higher expression of *ITGB1* mRNA was significantly associated with docetaxel resistance (n=15, r^2=0.66, P=0.0110). Suppression of *ITGB1* expression using siRNA sensitized the TE-2 cells to docetaxel. These data suggest that overexpression of *ITGB1* may be related to resistance to chemotherapy and that targeting *ITGB1*, particularly in patients on docetaxel therapy, may enhance the effect of chemotherapy in patients with ESCC.

Introduction

In Japan, >11,000 patients die of esophageal cancer every year, accounting for ~3% of Japanese cancer deaths; esophageal cancer is the sixth highest cause of cancer death in Japanese males. The treatment of advanced esophageal squamous cell carcinoma (ESCC) includes surgery, radiotherapy, chemotherapy, or more commonly a combined treatment regimen. Compared with radiotherapy alone, cisplatin-based chemoradiotherapy has been shown to improve the survival of patients with ESCC. Systemic chemotherapy has significantly reduced the incidence of distant metastasis and local recurrence, but its effect on overall survival has been less than satisfactory and newer drugs are currently being tested.

Docetaxel is a taxane that shows significant antitumor effects. It has shown clinical activity in a wide spectrum of solid tumors, including those of the breast, lung, ovary and prostate (1,2). Data concerning the biological effects and mechanisms of docetaxel on ESCC cells *in vitro* and *in vivo* are limited.

Herein, we have used microarray analysis to identify that the gene *ITGB1*, which encodes *ITGB1*, was specifically overexpressed in the cell lines that showed resistance to docetaxel. Integrins are cell surface adhesion receptors composed of α and ß subunits; they mediate cell-extracellular matrix and cell-cell interactions (3). *ITGB1* transduces biochemical signals from the extracellular environment, particularly those involved in growth, differentiation, invasiveness and metastatic potential of malignant cells (4,5). Altered expression of integrins has been reported to be involved in tumor suppression and progression (6-8). Here, we report that *ITGB1* expression might be related to tumor cell resistance to chemotherapy; thus, *ITGB1* might provide a therapeutic target, particularly in patients on docetaxel.

Materials and methods

Docetaxel and cell lines. Docetaxel (Sanofi-Aventis) was suspended in RPMI-1640 (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS). Serial dilutions were prepared to achieve final docetaxel concentrations of 0.05, 5, 1, 2.5, 5, 10, 25 and 50 ng/ml. The ESCC cell lines TE-1-5, TE-8-15, KYSE140 and KYSE150 were grown in RPMI-1640 containing 10% FBS. Giemsa staining, clonogenicity assays and small interfering RNA (siRNA) transfection were performed at 70-80% confluence.

Chemosensitivity. Cells were seeded into 96-well microtiter plates containing 100 µl culture medium at a concentration of

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$1x10^4$ cells/well. After 24 h, the cells were treated with docetaxel at concentrations of 0.05, 5, 1, 2.5, 5, 10, 25, and 50 ng/ml. All dilutions were performed in the culture medium. The total volume within each well was brought to 200 μl using growth medium and the plates were incubated at 37˚C under 5% CO_2 for 72 h. Further, 20 μl/well of the cell proliferation reagent WST-1 (Roche Applied Sciences, Mannheim, Germany) was added to 200 μl of the cell culture medium and held for 3 h. The absorbance of the samples was measured at 450 and 690 nm using a bichromatic ELISA reader. Relative proliferation was defined as the ratio of absorption measured in the docetaxel-treated cells (A) to that in the untreated control cells (A0). All experiments were performed in triplicate. IC_{50} values were calculated from plots of drug concentration vs. the proportion of cells that survived.

Oligonucleotide microarray. In order to reduce experimental errors, a set of oligonucleotide microarray slides containing a duplicate set of 34,594 spots was used to analyze each expression profile. Total RNA was isolated using the RNeasy mini kit (Qiagen, Chatsworth, CA) and digested by RNase-free DNase I (Nippon Gene Co., Tokyo, Japan) according to the recommendations of the manufacturers. We then performed T7-based RNA amplifications and preparations of cDNA probes using 5 μg of the total RNA, as described elsewhere (9,10). Amplified RNA (2.5 μg) from each cell line was labeled with Cy5-dCTP (Amersham Pharmacia Biotech, Uppsala, Sweden) and an equal amount of amplified RNA from a pool of the total RNA of the normal human esophageal mucosa cell line Het-1A (Invitrogen, Carlsbad, CA) was labeled with Cy3-dCTP (Amersham Pharmacia Biotech). Hybridization, washing and scanning were performed as described elsewhere (11,12). The intensity of each duplicated signal was evaluated photometrically using the ArrayVision computer program (Imaging Research, Inc., St. Catharines, Ontario, Canada). To normalize the mRNA in the cell lines, the Cy5:Cy3 ratio of each expressed gene was adjusted such that the mean Cy5:Cy3 ratio of Het-1A was 1.0. Subsequently, the duplicated spots on each slide were averaged (9,11,12). In addition, a cut-off value for each expression level was automatically calculated by variance analysis and data with low signal intensities were excluded from further investigation.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA (2 μg) was converted to cDNA using the GeneAmp RNA PCR core kit (Applied Biosystems, Tokyo, Japan), according to the manufacturer's instructions. qRT-PCR amplification of the cDNA template corresponding to 20 ng of total RNA was performed using Taq Man PCR Universal PCR Master Mix (Applied Biosystems, Foster City, CA) in an ABI PRISM 7500 sequence detection PCR system (Applied Biosystems). The PCR conditions were as follows: 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles each of 95˚C for 15 sec and 60˚C for 1 min. *ITGB1* Taq Man probes were synthesized from sequences in exons 10, 11 and 12 (Assays-on-Demand Gene Expression System, *ITGB1* assay ID: Hs00236976_m1; Applied Biosystems). Expression levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (Assays-on-Demand Gene Expression Systems, assay ID: Hs99999905_m1; Applied Biosystems).

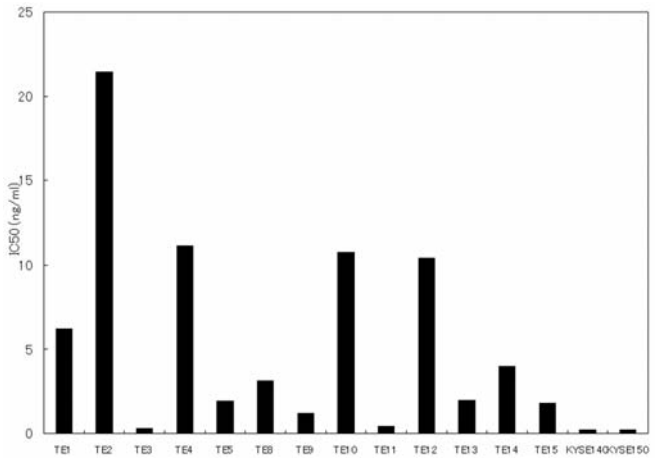
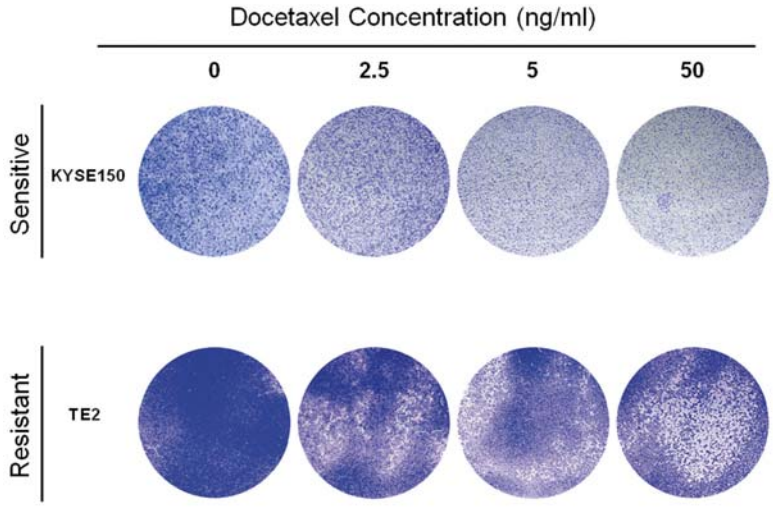


Figure 1. Docetaxel IC_{50} of 15 esophageal squamous cell carcinoma cell lines. Cells were exposed to graded dilutions of docetaxel and cell survival was determined using an MTT assay in triplicate; the mean of the assay values is shown.

Western blotting. Cells propagated in three-dimensional IrECM were first isolated with ice-cold phosphate-buffered saline/EDTA [0.01 mol/l sodium phosphate (pH 7.2) containing 138 mmol/l sodium chloride and 5 mmol/l EDTA]; then lysed in a radioimmunoprecipitation assay buffer. Equal amounts of protein were loaded onto reducing SDS gels. After transfer onto a nitrocellulose membrane (Invitrogen), blots were blocked with 5% non-fat milk and probed. Primary antibodies used include *ITGB1*, clone 18 (1:2500; BD Transduction Laboratories, Lexington, KY), α-PCNA (1:1,000; Santa Cruz Biotech, Santa Cruz, CA). Blots were washed, incubated with secondary antibody (Mouse IgG1) and exposed to X-rays.

siRNA transfection and cell proliferation assay. *ITGB1* siRNA (M-004506-00 Dharmacon, Lafayette, Co.) and control (siControl™ Non-targeting pool) transfections were performed using human T-cell nucleofector kits (Amaxa, Cologne, Germany) according to the manufacturer's instructions. The cells were seeded into 96-well plates in complete culture medium at a density of $5x10^4$ cells/well. After 24 h, medium was added with or without docetaxel (0.05, 5, 1, 2.5, 5, 10, 25 and 50 ng/ml). After 72 h, cell proliferation was assessed using an MTT assay (CellTiter 96; Promega, Madison, WI). Cell proliferation was determined as the ratio of the absorbance of docetaxel-treated cells to that of the control cells seeded with non-target siRNA. Each assay was performed in triplicate.

Statistical methods. The Graphcel software program was used to determine IC_{50} values. Statistical analyses were performed using the Mann-Whitney U test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between variables were determined by



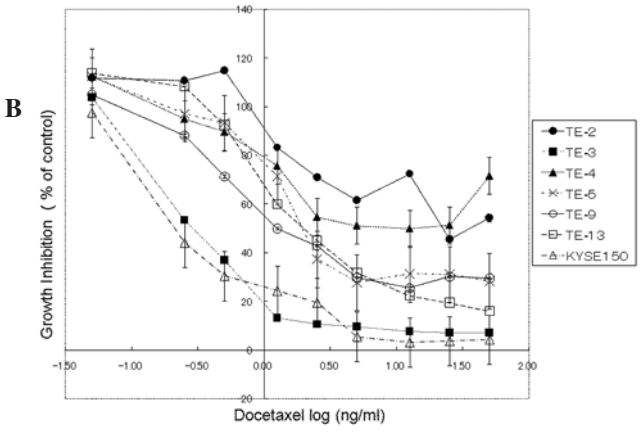
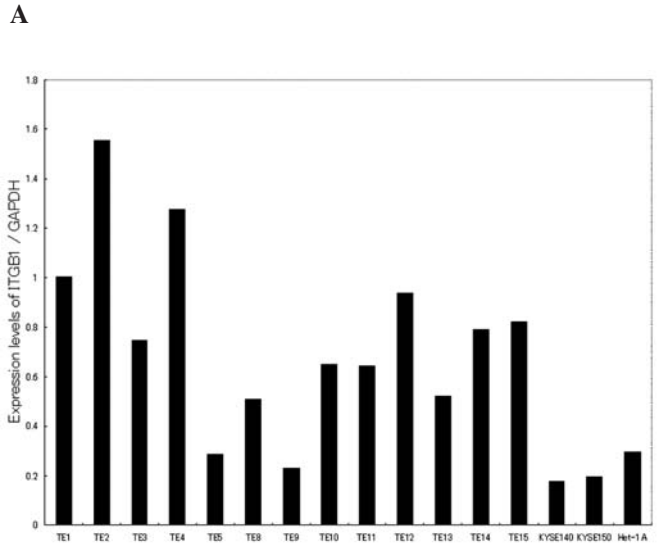


Figure 2. The sensitivity of two esophageal squamous cell carcinoma cell lines to docetaxel (A) Cells were cultured in medium containing the indicated concentrations of docetaxel and cell viability was measured at 72 h. Each well was stained with Giemsa. There were fewer colonies of the sensitive cell line (KYSE150) than the resistant cell line (TE-2) at docetaxel concentrations >2.5 ng/ml. Docetaxel IC_{50} of esophageal squamous cell carcinoma cell lines. (B) Cells were incubated with varying concentrations of docetaxel for 72 h and cell viability was determined using the MTT assay. The data shown are the mean (standard deviation) of triplicate cultures. The standard deviations were <10% of the mean.

means of a simple linear regression. The StatView software package (Abacus Concepts Inc., Berkeley, CA) was used for all analyses and $P<0.05$ was considered significant.

Results

Chemosensitivity of esophageal cancer cell lines to docetaxel. We used an MTT assay to determine the sensitivity (IC_{50}) of 15 esophageal cancer cell lines to docetaxel (Fig. 1). TE-2 was the most resistant cell line, followed by TE-4, TE-10, TE-12 and TE-1. Other cell lines showed moderate to high sensitivity (IC_{50} <5 ng/ml). The clonogenic potential of each cell line after docetaxel treatment varied and Fig. 2A illustrates two representative cell lines, the sensitive KYSE150 and the resistant TE-2. Fig. 2B shows the response of eight representative cell lines to varying concentrations of docetaxel measured in three separate experiments, each time in triplicate. Of the cell lines shown, three were sensitive (TE-3, KYSE140 and



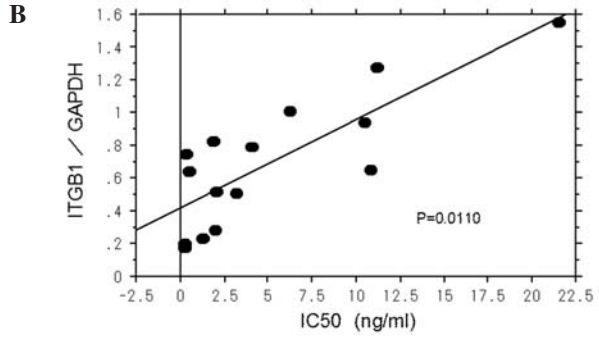


Figure 3. Expression of *ITGB1* mRNA in esophageal cancer cell lines and in a normal esophageal mucosal cell line, examined by quantitative RT-PCR. (A) Expression levels were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Correlation between *ITGB1* mRNA expression and resistance to docetaxel in 15 esophageal cancer cell lines. (B) The cell line with the highest *ITGB1* mRNA expression showed the highest resistance to docetaxel. The correlation was significant (P=0.0110).

Table I. Highly expressed genes.

Gene name	Map	EMBL	Gene name	Map	EMBL
EPLIN	12q13	BC010664	CYB5-M	16q22.1	BC004373
KRT6A	12q12-q13	L42599	SLC25A4	4q35	BC008664
DDX26	13q14.12-q14.2	AF097645	PERLD1	17q12	AF217980
IFNA2	9p22	A04970	C14orf124	14q11.2	AF226050
KRT5	12q12-q13	M19723	DXYS155E	Xp22.32	M99578
DDR2	1q12-q23	X74764	GOLPH2	9q21.33	
AK075542					
KISS1	1q32	AY117143	FLJ20308	17p11.2	
AK000315					
TM4SF1	3q21-q25	BC010166	S100P	4p16	BC006819
KRT6IRS	12q13.13	AJ308599	RP42	3q26.3	AF456425
HMS	11q14.1-q14.3		RGS2	1q31	BC007049
S100A2	1q21	BC002829	C17orf37	17q12	BC006006
DSC3	18q12.1	AF293359	GPR110	6p12.3	
AK026337					
CD44	11p13	AY101193	TFDP2	3q23	U35117
CAV1	7q31.1	BC009685	NCOR1	17p11.2	AB019524
DST	6p12-p11	U04850	PAPSS2	10q23-q24	BC009894
KRT5	12q12-q13	M19723	DAF	1q32	
AY055759					
COL17A1	10q24.3	AL138761	QSCN6	1q24	U97276
SFN	1p36.11	BC023552	PDCD8	Xq25-q26	AF131759
RPS7	2p25	X56846	HPGD	4q34-q35	J05594
BMP7	20q13	BC004248	ALOX15B	17p13.1	AF468052
TMEM40	3p25.2	AK092470	HSPC171	16q22.1	BC003080
CTSC	11q14.1-q14.3	U79415	CLDN1	3q28-q29	BC012471
RBPMS	8p12-p11	D84111	C14orf147	14q13.1	BC016805
COL5A2	2q14-q32	M11718	MANSC1	12p13.2	
AK023622					
ERBB2	17q11.2-q12	X03363	C11orf2	11q13	BC017438
AGR2	7p21.3	AF115926	BENE	2q13	U17077
NMU	4q12	BC012908	FLII	17p11.2	U01184
KRT7	12q12-q13	BC002700	DUSP6	12q22-q23	BC037236
C14orf135	14q23.1	BC033843	BAG1	9p12	AF116273
AKAP13	15q24-q25	AB055890	LY6D	8q24-qter	U66837
YME1L1	10p14	BC007795	ITGB1	10p11.2	U33881
PTGES	9q34.3	BC008280	LCN2	9q34	S75256

KYSE150), four were resistant (TE-2, TE-4, TE-10 and TE-12) and the remaining eight cell lines exhibited intermediate sensitivity to docetaxel (Fig. 2B). TE-2 was the most resistant to docetaxel (IC_{50} >20 ng/ml).

Microarray analysis of genes differentially expressed in cell lines sensitive or resistant to docetaxel. Next, a microarray analysis was performed in order to identify the genes whose expression may confer resistance to docetaxel. Total RNA was extracted from each cell line and the reverse transcriptase reaction was carried out. Table I shows the genes whose expression was high in the docetaxel-resistant TE-2 cell line and low in the other cell lines. We focused on *ITGB1* for further analysis.

Expression of ITGB1 in esophageal cancer cell lines and its relationship with docetaxel sensitivity. We examined *ITGB1* mRNA expression in the 15 cell lines and in the normal human esophageal mucosa cell line Het-1A by quantitative PCR. *ITGB1* expression was found to be higher in many esophageal cancer cell lines than in Het-1A. *ITGB1* mRNA expression was very high in TE-1, TE-2 and TE-4 (Fig. 3A).

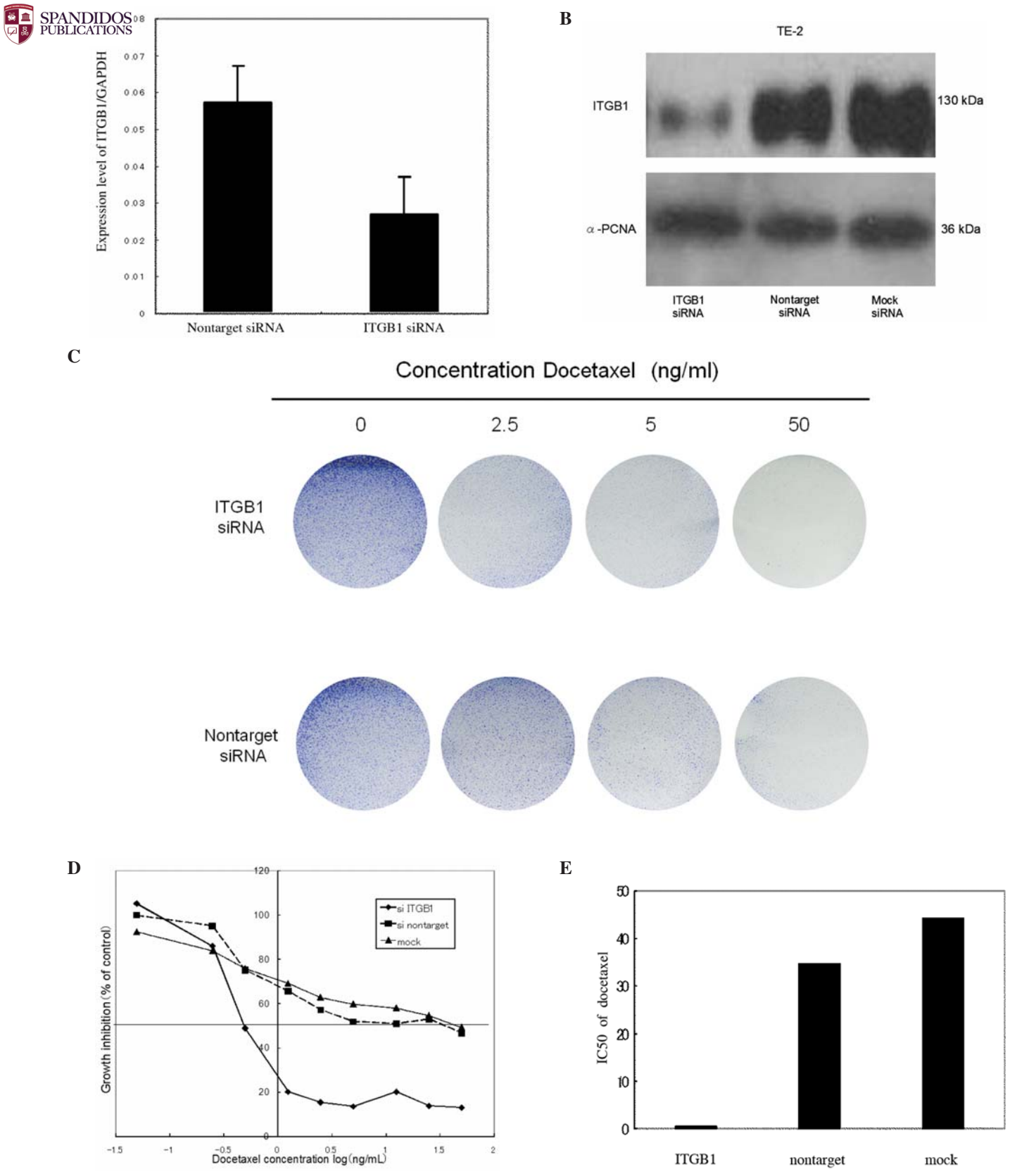


Figure 4. Expression of *ITGB1* mRNA in TE-2 was down-regulated by a siRNA. (A) Expression levels were measured by real-time RT-PCR. The mean and standard error of the triplicate assay are shown. Western blot analysis of *ITGB1* protein expression in the siRNA-treated TE-2 cells. (B) siRNA of *ITGB1* down-regulated the expression of *ITGB1* protein in TE-2; however, it had little effect on α-PCNA. Non-specific siRNA (non-target siRNA) or mock transfection (mock siRNA) had no effect. Sensitivity to docetaxel of TE-2 cells treated with siRNA of *ITGB1*. (C) Cells that were treated with siRNA of *ITGB1* or seeded with non-target siRNA were incubated with varying concentrations of docetaxel for 72 h and each well was stained with Giemsa. Sensitivity to docetaxel of TE-2 cells treated with siRNA of *ITGB1*. (D) Cells treated with or without the siRNA of *ITGB1* or non-target siRNA were incubated with varying concentrations of docetaxel for 72 h and cell viability was determined using an MTT assay. The data shown are the mean of triplicate cultures. The standard deviations were <10% of the mean. (E) Docetaxel IC_{50}s of ESCC cell lines, calculated using the data shown in (D). Cells were exposed to varying concentrations of docetaxel. Cell survival was determined using the MTT assay.

It was highest in the TE-2 cell line, which was the most resistant to docetaxel. Regression analysis revealed a strong positive correlation between expression of *ITGB1* mRNA and docetaxel IC_{50} in ESCC cell lines. The coefficient of correlation was 0.813 (Fig. 3B).

Inhibition of ITGB1 restores chemosensitivity of esophageal cancer cells to docetaxel. We used the siRNA technique to down-regulate *ITGB1* mRNA in the TE-2 cell line (Fig. 4A), which was chosen because it was the most resistant to docetaxel and had the highest expression of *ITGB1*. Western blotting proved that *ITGB1* protein level was also reduced by siRNA (Fig. 4B). The down-regulation of *ITGB1* had a pronounced effect on the sensitivity of the cell to docetaxel. At the same concentration of docetaxel, cells treated with *ITGB1* siRNA had fewer colonies than cells treated with non-target siRNA (Fig. 4C). This enhancement of sensitivity by siRNA of *ITGB1* was further confirmed using the quantitative MTT assay (Fig. 4D). Fig. 4E shows that the IC_{50} of TE-2 cells treated with *ITGB1* siRNA was significantly lower than untreated cells, as calculated from Fig. 4D.

Discussion

Despite the fact that many different chemotherapeutic agents and regimens have been developed to treat advanced ESCC, there has been no significant increase in patient survival. Therefore, novel chemotherapeutic strategies are necessary. Docetaxel is an antimicrotubulin agent that has antiproliferative and suppressive effects on cancer cells. It is known that in the basic mechanism, it binds to tubulin and disturbs the equilibrium between microtubule assembly and disassembly during mitosis (13). Stabilization of microtubules by docetaxel impairs mitosis and exerts an anticancer effect in tumors (13). In addition to its effects on microtubules, docetaxel also induces apoptosis with down-regulation of bcl-xL and bcl-2 and up-regulation of p21WAF1 and p53 (14,15). Integrins, heterodimeric transmembrane receptors composed of α and ß subunits, can be found in focal adhesions. In ESCC patients, no clear correlation has been demonstrated between tumor formation, invasion and *ITGB1* expression. In mice, however, a crucial role of *ITGB1* in tumor formation and metastasis has been demonstrated. *ITGB1* is aberrantly expressed in human breast carcinomas and has been shown to play a central role in growth, apoptosis, invasion and metastasis (16-20). In addition to its role in cancer progression, an emerging body of evidence indicates that *ITGB1* signaling plays a significant role in mediating the resistance to cytotoxic chemotherapies by enhancing cell survival in hematologic, lung and breast malignancies (21-24). Inhibition of *ITGB1* has also been shown to abolish metastasis in gastric and breast cancer models (25-28). Our results suggest that integrin signaling is an important survival pathway in cancer cells treated with docetaxel.

We have found here that *ITGB1* is specifically up-regulated in an esophageal cell line with high resistance to docetaxel (Fig. 3A) and that *ITGB1* mRNA expression positively correlates with docetaxel resistance (Fig. 3B). When *ITGB1* expression was down-regulated using siRNA (Fig. 4A and B), cell sensitivity to docetaxel was apparently restored (Fig. 4D and E). These results indicate that expression of *ITGB1* may be an important determinant of the resistance of a cell to docetaxel.

It has previously been reported that *ITGB1* signaling is an important survival pathway in drug-induced apoptosis of breast cancer cells and that activation of this pathway may contribute to the development of drug resistance (21). There have been no previous studies of a relationship between *ITGB1* and esophageal cancer. We are also unaware whether *ITGB1* plays a role in cell response to other chemotherapeutic reagents, although the cell lines studied here showed distinct sensitivities to cis-diamminedichloride platinum (data not shown). The mechanisms by which *ITGB1* expression confers chemoresistance are unknown and future studies will aim to discover whether they are related to the function of integrins in cell adhesion. A complete understanding of the molecular events that mediate integrin-dependent survival of cancer cells may lead to new therapeutic tools for cancer treatment.

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