Cancer Science
Japanese Cancer Association JCA
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SIX1 maintains tumor basal cells *via* transforming growth factor-β pathway and associates with poor prognosis in esophageal cancer

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Key words

Epithelial-mesenchymal transition, esophageal cancer, prognosis, SIX1, transforming growth factor-β

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Funding information

Japan Agency for Medical Research and Development (Practical Research for Innovative Cancer Control), National Cancer Center Research and Development Fund, The Ministry of Education, Culture, Sports, Science and Technology of Japan, and The Princess Takamatsu Cancer Research Fund

Received April 14, 2016; Revised December 4, 2016; Accepted December 8, 2016

Cancer Sci **108** (2017) 216–225

doi: 10.1111/cas.13135

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant tumors. Although improvement in both surgical techniques and neoadjuvant chemotherapy has been achieved, the 5-year survival rate of locally advanced tumors was, at best, still 55%. Therefore, elucidation of mechanisms of the malignancy is eagerly awaited. Epithelial-mesenchymal transition (EMT) by transforming growth factor-β (TGF-β) has been reported to have critical biological roles for cancer cell stemness, whereas little is known about it in ESCC. In the current study, a transcriptional factor SIX1 was found to be aberrantly expressed in ESCCs. *SIX1* cDNA transfection induced overexpression of transforming growth factors (*TGFB1* and *TGFB2*) and its receptor (*TGFBR2*). Cell invasion was reduced by *SIX1* knockdown and was increased in stable *SIX1*-transfectants. Furthermore, the *SIX1*-transfectants highly expressed tumor basal cell markers such as *NGFR*, *SOX2*, *ALDH1A1*, and *PDPN*. Although mock-transfectants had only a 20% PDPN-high population, *SIX1*-transfectants had 60–70%. In two sets of 42 and 85 ESCC patients receiving surgery alone or neoadjuvant chemoradiotherapy followed by surgery, the cases with high SIX1 mRNA and protein expression level significantly showed a poor prognosis compared with those with low levels. These SIX1 high cases also expressed the above basal cell markers, but suppressed the differentiation markers. Finally, TGF-β signaling blockade suppressed ESCC cell growth in association with the reduction of PDPN-positive tumor basal cell population. The present results suggest that SIX1 accelerates self-renewal of tumor basal cells, resulting in a poor prognosis for ESCC patients.

Esophageal squamous cell carcinoma (ESCC) is one of the most common malignant tumors in Eastern Asia with extremely poor prognosis due to late presentation and rapid progression.[1] Neoadjuvant chemoradiotherapy (CRT) followed by surgery is the standard therapy in Western countries, whereas neoadjuvant chemotherapy followed by surgery and definitive CRT are the standard therapies in Japan.[2] Although improvement in both surgical techniques and neoadjuvant chemotherapy has been achieved, the 5-year survival rate of locally advanced tumors was, at best, still 55%.[3] Local recurrence, lymph node metastasis, and distant metastasis are major causes of such a poor prognosis. Therefore, elucidation of this mechanism is eagerly awaited.

Epithelial-mesenchymal transition (EMT), well-known to be critical to a defining structural feature of organ development, is involved in disease progression, including that of cancer.[4] Its induction can cause enhanced cellular motility and invasiveness which is essential for metastasis, and often leads to the acquisition of stem cell properties.[5] Stemness properties are characterized by apoptosis resistance, transient quiescence, and self-renewal capacities, all of which cause a poor prognosis through metastasis and drug resistance. Despite its definitive biological role for cancer progression, little is known about the cancer stem cells of ESCC compared to other solid tumor stem cells.[6]

Since the first report by Feinberg *et al.*, aberrant DNA hypometyhlations have been observed in several types of cancer and have also been reported to be associated with tumorigenesis and metastasis.[7] We investigated such aberrantly expressed genes in ESCC by comparative microarray profiling between normal and tumor tissues.[8] Consequently, we found some aberrantly expressed genes, including *SIX1*. Among SIX homeodomain transcription factors, SIX1, SIX2 and SIX4, have an important role in the expansion of progenitor cell populations during early embryogenesis,[9] and are known to be essential for the development of numerous organs.[10–12]

Particularly, SIX1 has reported to be overexpressed and associated with poor prognosis in various human cancers.[13–18] In breast cancer, SIX1 induces EMT- and stem cell-like phenotypes through the upregulation of transforming growth factor β (TGF-β) signaling.[18–20] In ESCC, we revealed that SIX1 also induces EMT, inhibits cell differentiation, and promotes self-renewal of cancer stem cells *via* TGF-β signaling, and that its inhibition causes the reduction of stem cell population and induction of cell death. Therefore, the SIX1-regulated TGF-β signaling pathway has a potential to be a therapeutic target in ESCC.

Materials and Methods

Tissue samples of ESCC and normal esophagus. Both esophageal cancer tissues and their matched noncancerous tissues were obtained with written informed consent from locally advanced ESCC patients who underwent esophagectomy at the National Cancer Center Hospital (Tokyo, Japan) and Hiroshima University Hospital (Hiroshima, Japan), and biopsy samples of locally advanced ESCC before treatment were provided by the National Cancer Center Hospital East (Kashiwa, Japan) after obtaining written informed consent from each patient and approval by the institutional review boards.

Cell culture. All ESCC-derived cell lines were cultured in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum, penicillin-streptomycin at 37°C, with 5% CO_2 in 95% humidified air.

Laser-captured micro-dissection (LCM). The human esophagus was embedded in TissueTek OCT medium (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands) and snap-frozen in liquid nitrogen. The cryostat sections (8 μm) were laser-microdissected with a PixCell II LCM system (Arcturus Engineering, Mountain View, CA, USA).

RNA extraction and microarray analysis. For total RNA isolation, surgical specimens and esophageal epithelial cells of mice were lysed by ISOGEN lysis buffer (Nippon Gene, Toyama, Japan), extracted with chloroform, and precipitated with a glycogen carrier in isopropanol. The mRNA was amplified by an efficient method of high-fidelity mRNA amplification developed by us, called TALPAT (T7RNA polymerase promoter-attached, adaptor ligation mediated, and PCR amplification followed by *in vitro* T7-transcription).

Reverse Transcription-PCR and quantitative real-time PCR. Ten micrograms of cRNA from 1 to 5 μg total RNA was prepared from the esophageal cancer cell lines and the surgical specimens of esophageal cancer by T7 transcription-mediated RNA amplification. Single stranded cDNAs were synthesized from 5 μg cRNA by use of First-strand synthesis kit (Amersham Biosciences, Piscataway, NJ, USA) with random hexamers. We performed RT-PCR by Accuprime PCR system (Invitrogen, Carlsbad, CA, USA). The thermal profile consisted of an initial denaturation at 95°C for 5 min followed by repetitions at 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. All of the genes from 50 ng of the cDNA template were amplified with multiple cycle numbers (20–50 cycles) to determine the appropriate conditions for obtaining semiquantitative differences in gene expression levels. Quantitative real-time PCR was performed by a Bio-Rad iCycler with iQ Syber Green Supermix (Bio-Rad, Hercules, CA, USA) as directed by the manufacturer. The value of 1/2N (N: the number of PCR cycles corresponding to the onset of the linear amplification of each gene product) was calculated as a relative mRNA expression level of each gene normalized to *ACTB*. Primers used for the study are listed in Supplementary Table S1.

Plasmid construct and transfection. The pCMV6-XL5 containing *SIX1* cDNA was purchased from OriGene Technologies (Rockville, MD, USA) and integrated into pcDNA3.1 vector (Invitrogen). 2×10^4 cells were inoculated, and then transfected with either pcDNA3.1-*SIX1*, or no insert of pcDNA3.1-mock using Lipofectamine 2000 reagents according to the manufacturer's protocol (Invitrogen). The cells were selected with 0.5 mg/mL G418 (Invitrogen) from 2 days post-transfection, and simultaneously harvested at 2 days after transfection for RT-PCR analysis. Cells were also subjected to limiting dilution in a 96-well plate for obtaining colonies. *SIX1* mRNA expression levels of the clones were examined by quantitative RT-PCR.

Immunohistochemical analysis. Specimens fixed in formalin and embedded in paraffin were cut into 4-μm sections, subsequently dewaxed, and dehydrated. Sections were blocked for DAKO protein block (DAKO, Carpinteria, CA, USA), and stained with primary antibodies against Six1 (1:100, Atlas antibodies, Stockholm, Sweden), and PDPN (1:50, Acris Antibodies GmbH, Herford, Germany) at 4°C overnight, followed by incubation with EnVsion + Dual Link System-HRP (DAKO). Subsequently, these sections were exposed by DAB for 5 min. The slides were counterstained with hematoxylin and then mounted.

Immunofluorescence staining. Cells were cultured on glass chamber slides, and then fixed with 4% paraformaldehyde, permeabilized with −20°C methanol and 0.5% Triton X-100/PBS, and blocked with 0.1M NH_4Cl, 10% fetal bovine serum and 2% bovine serum albmine in phosphate -buffered saline (PBS). Cells were incubated with primary antibody for PDPN (1:50, Acris Antibodies GmbH), and then incubated with Alexa488-conjugated anti-mouse IgG antibody (1:800, Invitrogen) and stained with DAPI.

siRNA transfection. Purchased siRNA (ID: s227324, Ambion, Austin, TX, USA) were transfected to ESCC cells using DharmaFECT (Thermo Fisher Scientific, Waltham, MA, USA) following the procedure recommended by the manufacturer. Quantitative RT-PCR and Matrigel invasion assay were carried out after siRNA treatment of ESCC cells.

Matrigel invasion assay. Invasion abilities of ESCC cells *in vitro* were measured by BD BioCoat Matrigel Invasion Chamber (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. The cells were trypsinized and transferred into wells at 24 h after siRNA transfection. After incubation for 24 h, the cells that passed through the filter into the lower wells were fixed, stained, and counted.

Flow cytometry analysis. Cells were harvested, and incubated with anti-PDPN antibody (1:100, Abcam, Cambridge, UK) or isotype control mouse IgG1 for 30 min on ice followed by PBS washing. Anti-mouse IgG-Alexa488 (1:800; Invitrogen) was used as a secondary antibody. Dead cells were labeled with propidium iodide and were excluded from the analysis. Flow cytometry analysis was performed using FACSCalibur (BD Biosciences).

Western blot. Cells were lysed in Laemmli Sample buffer (Bio-Rad, Hercules, CA, USA) containing DTT and 1% protease inhibitor cocktail. Nuclear extracts at 24 h after TGF-β (R&D Systems, Minneapolis, MN, USA) treatment or at 48 h after TGF-β receptor inhibitor (A-83-01, Wako, Tokyo, Japan) treatment were prepared by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). The samples were separated using 12% SDS-PAGE. Proteins were

transferred to polyvinylidene fluoride (PVDF) membrane and blocked with 5% membrane blocking agent (GE Healthcare, Buckinghamshire, UK) in PBS, and probed with anti-SIX1 antibody (1:250, Acris Antibodies GmbH, Herford, Germany) or anti-phospho-smad2/3 antibody (1:500, Cell Signaling Technology, Danver, MA, USA) at 4°C overnight, anti-α tubulin antibody (1:1000; Santa Cruz, Dallas, Tx, USA) at room temperature for 1 h or anti-β actin antibody (1:2000; Cell Signaling Technology) at room temperature for 2 h, then washed and incubated with HRP-conjugated anti-rabbit immunoglobulin (DAKO) at room temperature for 2 h. Immunoreactive protein bands were identified with Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific).

Treatment of TGF-β receptor inhibitor. Cells were seeded at a density of 5×10^5 cells per 100 mm dish with RPMI-1640 containing 10% fetal bovine serum (FBS). The following day, cells were treated with 1 μM or 10 μM A-83-01 and the medium was changed every 2 days until 7 days post-treatment. Cells were trypsinized, counted the viable cells, and prepared for flow cytometry and RT-PCR.

Animal experiments. *SIX1*-transfectants were transplanted into scid/scid mice by subcutaneous injection of 5×10^6, 1×10^7, or 2×10^7 cells under anesthesia. Briefly, the cells were resuspended in a 6:4 mixture of medium and Matrigel (BD Biosciences). Tumor growth of total six mice was observed for 7 weeks. All animal studies were approved by the Animal Experiments Committee at the National Cancer Center Research Institute.

Statistical analysis. Clinical samples were classified into two groups for each analysis: samples in which *SIX1* expression was higher than the mean (SIX1 "high"), and lower than the mean (SIX1 "low"). Each dataset was analyzed separately and *P*-values were calculated by log-rank analysis. The accepted level of significance was $P < 0.05$.

Results

Expression of *SIX1* in esophageal squamous cell carcinomas (ESCCs). We first investigated *SIX1* and *PDPN* mRNA expression in eight ESCC cell lines (TE1, TE3, TE5, TE6, TE8, TE10, T.Tn, and KYSE510) and normal esophageal epithelium that was divided into basal, epibasal, and differentiated cell layers by laser-captured microdissection. *SIX1* mRNA was detected in ESCC cell lines by 30 cycles of PCR, while

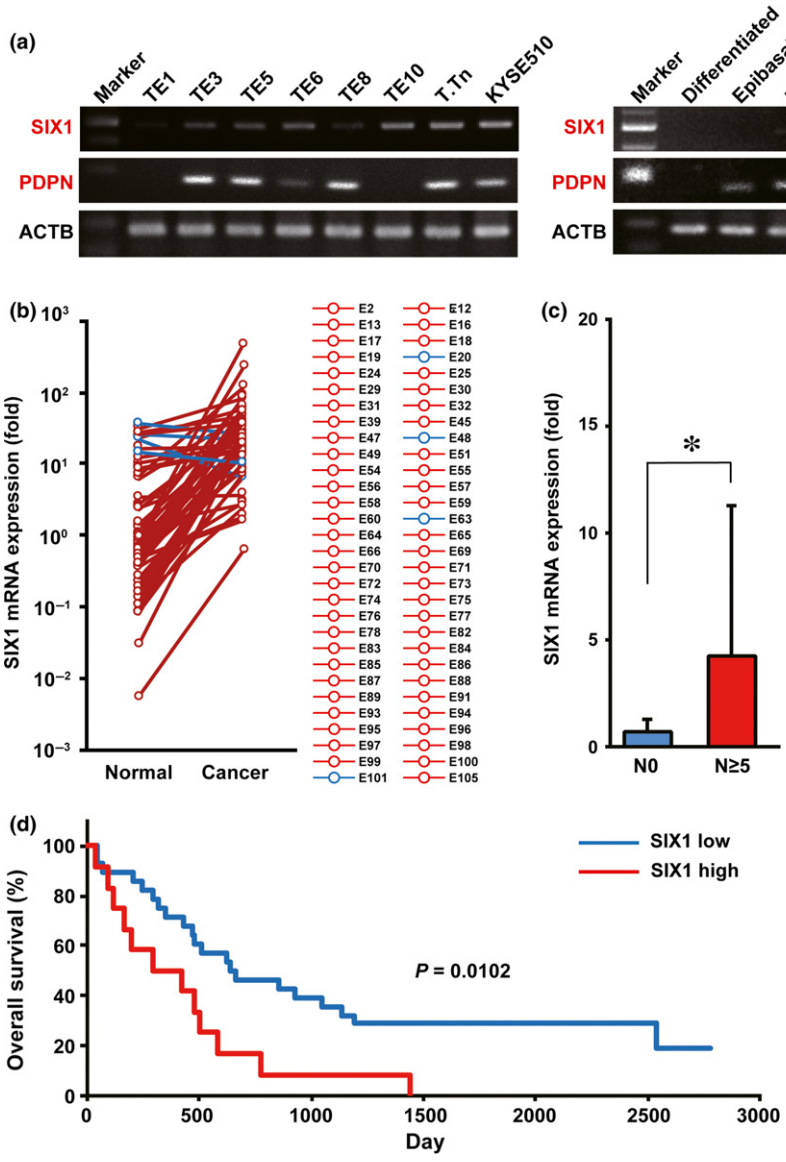

Fig. 1. Selective expression of SIX1 in esophageal squamous cell carcinoma (ESCC). (a) Semi-quantitative RT-PCR analyses of *SIX1* and *PDPN* in eight ESCC cell lines (30 PCR cycles for *SIX1* and *PDPN*) and three layers (Differentiated, Epibasal, and Basal cell layer) of normal esophageal mucosa (50 PCR cycles for *SIX1* and 30 PCR cycles for *PDPN*). (b) Quantitative RT-PCR of *SIX1* in 60 pairs of primary ESCCs and the matched normal tissues. (c) Quantitative RT-PCR of *SIX1* in 42 ESCC specimens classified with no lymph node metastasis (N0, $n = 12$) and high metastasis ($N \geq 5$, $n = 30$). Data are mean ± SD from three independent experiments. * $P < 0.05$. (d) Overall survival of 42 ESCC patients stratified by *SIX1* expression level. *P*-values are calculated by log-rank test.

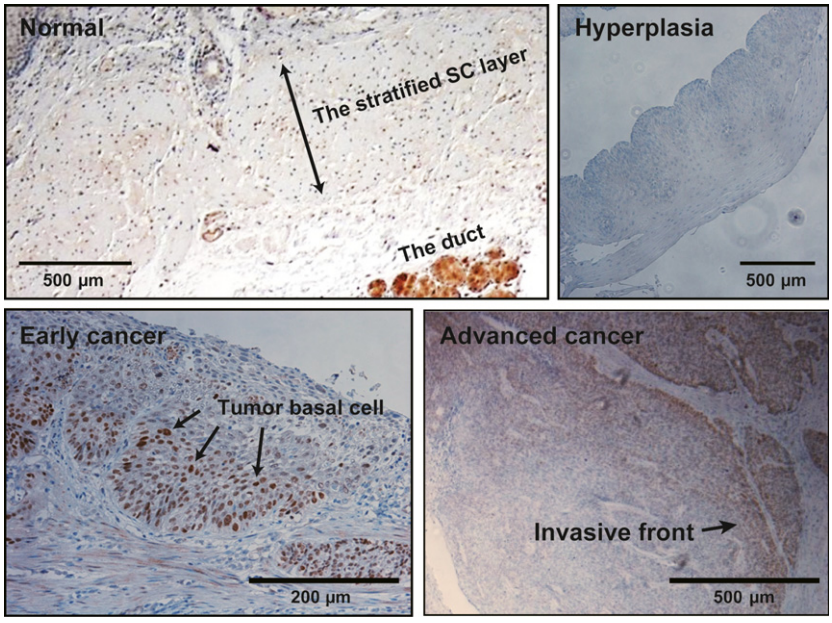

Fig. 2. Expression of SIX1 in normal esophagus, hyperplasia, early esophageal squamous cell carcinoma (ESCC) and advanced ESCC. Representative images of immunohistochemistry for SIX1 in normal esophagus (left top), hyperplasia (right top), early cancer (left bottom), and invasive front in advanced ESCCs (right bottom). Section is counterstained with hematoxylin. SC, squamous cell. Scale bars represent 500 μm and 200 μm.

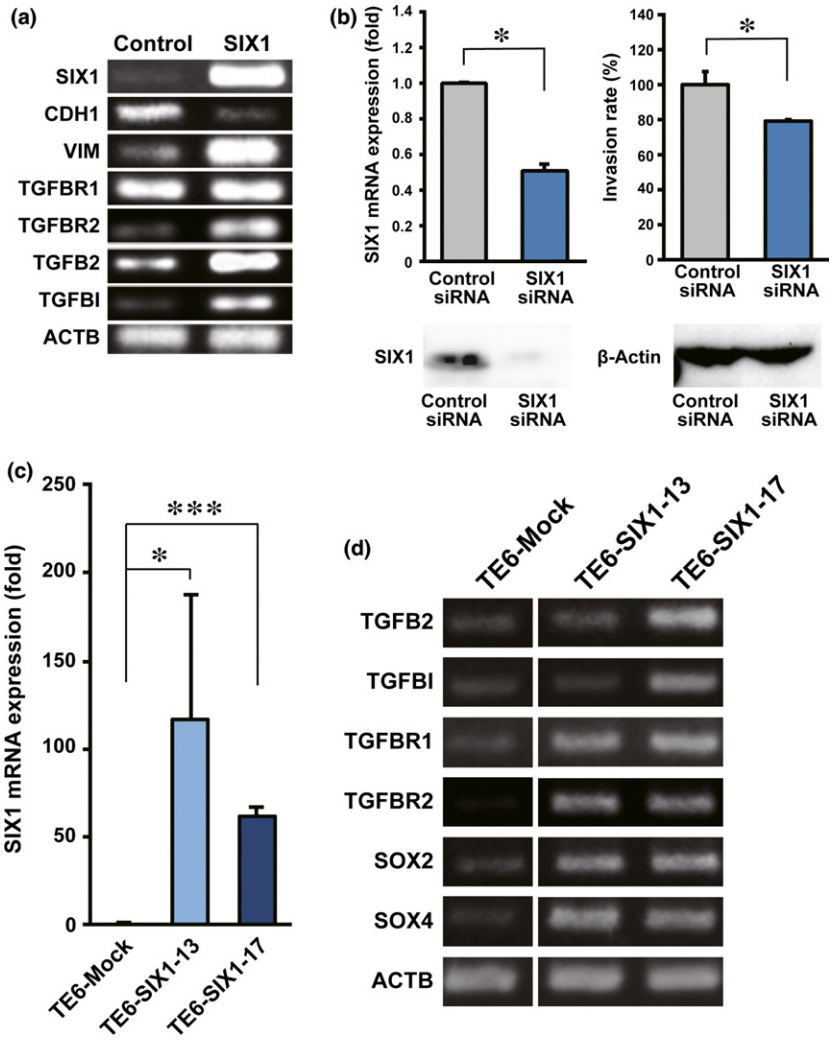

Fig. 3. SIX1 regulated genes related to epithelial-mesenchymal transition (EMT) by transforming growth factor-β (TGF-β) pathway. (a) Semi-quantitative RT-PCR of *SIX1*, an epithelial marker (*CDH1*), a mesenchymal marker (*VIM*), TGF-β related genes (*TGFB2* and *TGFBI*), and their receptors (*TGFBR1*and *TGFBR2*) in TE1 cells transiently transfected with human *SIX1* cDNA or mock vector. Total RNA is recovered from cells at 48 h after transfection. A size marker lane is cropped from a photograph. (b) Matrigel invasion assay of TE3 cells transiently knockdown by *SIX1* siRNA (upper right). Cells are transferred into matrigel at 24 h after transfection of *SIX1* or control siRNA. Migrated cells are counted at 24 h after transfer. Data are mean ± SD from three independent experiments. Data of quantitative RT-PCR (upper left) and Western blotting (lower) of SIX1 are also shown. (c) Quantitative RT-PCR analysis of *SIX1* in *SIX1*-stable transfectants (TE6-SIX1-13 and TE6-SIX1-17) and mock-transfectants (TE6-Mock). Data are mean ± SD from three independent experiments. * $P < 0.05$, *** $P < 0.005$. (d) Semi-quantitative RT-PCR of SIX1 and TGF-β signal-related genes (*TGFB2*, *TGFBI, TGFBR1, TGFBR2*, *SOX2, and SOX4*) in the two *SIX1*-transfectants and mock-transfectants. Lanes of a size marker, positive control (left of TE6-Mock), and another unstable clone (right) are cropped from a photograph. Photographs of five full uncut gels were shown in Fig. S3.

that was detected in normal basal cell layer only by more than 50 cycles. Accordingly, we concluded that *SIX1* was activated in most of the ESCC cell lines, but suppressed in normal epithelium (Fig. 1a). Correlation in mRNA expression between *SIX1* and *PDPN*, which is a basal and ductal cell marker, was observed in ESCC cell lines except TE10

(Fig. 1a). Next, we analyzed the mRNA expression of *SIX1* in 60 pairs of primary ESCCs and its corresponding normal portions by real-time RT-PCR, and found more than 90% of ESCCs showed aberrant expression of *SIX1* (Fig. 1b, red lines). We also investigated the relationship between the *SIX1* mRNA level and the status of lymph node metastasis in 42 surgically resected specimens of locally advanced ESCC (stage II and III). The result showed that *SIX1* mRNA expression was significantly higher in 30 cases that had more than five metastatic lymph nodes than in 12 cases with no metastatic lymph nodes (Fig. 1c). Accordingly, the high *SIX1* expression was significantly correlated with the poor prognosis of ESCC patients received by surgery alone (Fig. 1d). We also performed immunohistochemical analyses to evaluate SIX1 protein expression in normal mucosa, hyperplasia, and cancer. Consistent with the above mRNA expression analyses, SIX1 was expressed neither in normal stratified squamous cell layer nor hyperplasia, but was expressed in the normal duct and tumor basal cells of an early ESCC (Fig. 2). Of particular note, SIX1 was positively stained at basal layers or the invasive front in advanced ESCCs (Fig. 2), indicating its involvement in invasion and cell motility. Based on these results, we focused on the impact of SIX1 expression on epithelial-mesenchymal transition (EMT) and on tumor basal cell or cancer stem cell phenotype, since SIX1 has been reported to be an EMT inducer in breast cancer.[19, 20]

SIX1 induces TGF-β signal-related genes and undifferentiated basal cell phenotype in ESCC cells. To investigate the biological role of SIX1 in ESCCs, we performed transient transfection of *SIX1* cDNA to TE1 cells. RT-PCR analysis revealed that overexpression of *SIX1* led to upregulation of a mesenchymal marker, vimentin (*VIM*), and downregulation of an epithelial marker, E-cadherin (*CDH1*) (Fig. 3a). Interestingly, a series of the TGF-β related genes (*TGFBI* and *TGFB2*) and their receptor genes (*TGFBR1* and *2*) were highly expressed accompanying *SIX1* overexpression (Fig. 3a). Furthermore, the invasive ability of TE3 cells was significantly reduced by siRNA-based knockdown of *SIX1* mRNA and protein (Fig. 3b). These results suggest that SIX1 promotes tumor invasion through EMT induction *via* TGF-β signaling in ESCC.

Next, we chose TE6 cell line with low invasion ability (Fig. S1) and established two clones stably expressing *SIX1* (TE6-SIX1-13 and TE6-SIX1-17) by limiting dilution (Fig. 3c). Consistent with transient transfection of *SIX1* cDNA to TE1 with a quit low *SIX1* expression (Fig. 3a) and with that of *SIX1* siRNA to TE3 with a significant *SIX1* expression

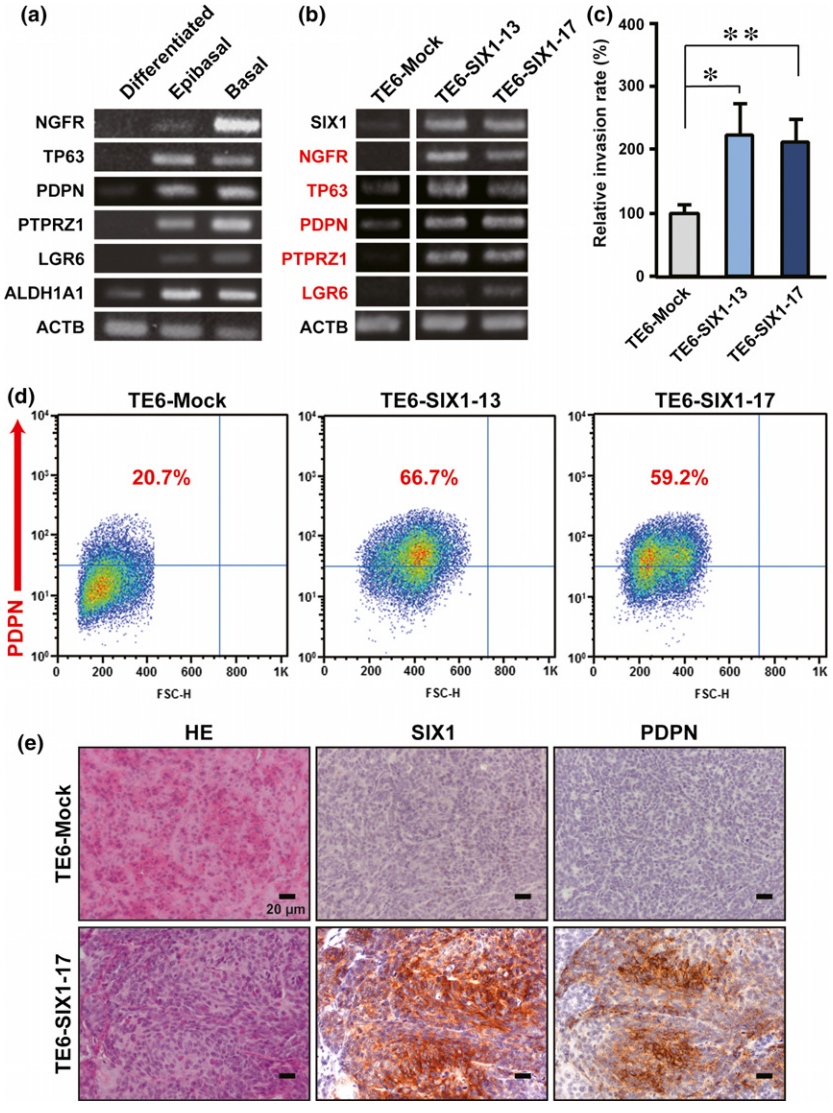

Fig. 4. Continues expression of SIX1 alter esophageal squamous cell carcinoma (ESCC) cell to tumor basal phenotype. (a) Semi-quantitative RT-PCR of *SIX1* and normal basal cell or stem cell markers (*NGFR*, *TP63*, *PDPN*, *PTPRZ1*, *LGR6,* and *ALDH1A1*) in three layers (differentiated, epibasal, and basal cell layer) of normal esophageal mucosa. A size marker lane is cropped from a photograph. (b) Semi-quantitative RT-PCR of *SIX1* and the six above stem cell markers in two *SIX1*-transfectants (TE6-SIX1-13 and TE6-SIX-17) and mock-transfectants (TE6-Mock). Lanes of a size marker, positive control (left of TE6-Mock), and another unstable clone (right) are cropped from a photograph. Photographs of four full uncut gels were shown in Fig. S6. (c) Matrigel invasion assay of *SIX1*-transfectants. The migrated cells counted at 24 h after transfer into wells. Data are mean ± SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$. (d) Flow cytometry analysis of *SIX1*-transfectants for PDPN expression. Cells are double-stained by anti-PDPN antibody and PI. Only viable cells are analyzed. (e) Immunohistochemical study of xenografted tumors of TE6-Mock and TE6-SIX1-17. HE (left), anti SIX1 antibody (middle) anti-PDPN antibody (right). Scale bars represent 20 μm.

(Fig. S2), both TE6-SIX1-13 and TE6-SIX1-17 transfectants highly expressed TGF-β signal-related genes (TGF-β ligands, their receptors and downstream transcription factors SOXs) compared to the empty vector-transfected mixed clones, termed TE6-Mock (Fig. 3d). Lanes of a size marker, positive control (left of TE6-Mock), and another unstable clone (right) are cropped from a photograph. Photographs of five full uncut gels were shown in Fig. S3. We also confirmed no difference in their expression levels between the parental TE6 cell line and the TE6-Mock (Fig. S4). Since the *CDH1* induction and *VIM* reduction were shown by transient *SIX1* cDNA transfection (Fig. 3a), it should be noted that the reduction of *CDH1* mRNA and the induction of an EMT marker (*VIM*) and five EMT regulators (*SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, and *TWIST2*) by SIX1 were not observed in the two *SIX1*-transfectants (Fig. S5). These data suggested that a main role of SIX1 in ESCC is the induction of TGF-β signal-related genes.

Furthermore, these two cell lines also highly expressed many markers for undifferentiated esophageal epithelial cells (*i.e.* basal and epibasal cells) such as *NGFR*, *SOX2*, *TP63*, *PDPN*, *PTPRZ1*, and *LGR6* (Fig. 4a,b). Lanes of a size marker, positive control (left of TE6-Mock), and another unstable clone (right) are cropped from a photograph. Photographs of four full uncut gels were shown in Fig. S6. In accordance with a result of *SIX1* knock down (Fig. 3b), both *SIX1*-transfectant showed higher invasive abilities than that of mock-transfectants (Fig. 4c). To explore the relationship of SIX1 and cancer stem cells, we selected PDPN from genes selectively-expressed in undifferentiated esophageal epithelial cells, since it has been reported that PDPN is a cancer stem cell marker of squamous cell carcinoma.[21,22] Flow cytometry analysis showed both of the *SIX1*-transfectants contained a high PDPN-positive fraction (66.7% and 59.2%, respectively) compared to that of TE6-Mock (20.7%) (Fig. 4d). We also assessed the tumorigenicity of mock- and *SIX1*-transfectants in immunodeficient mice. As a result, only the mouse transplanted TE6-SIX1-17 cells, which stably over-expressed SIX1 by multiple passages, developed a massive tumor (data not shown). Furthermore,

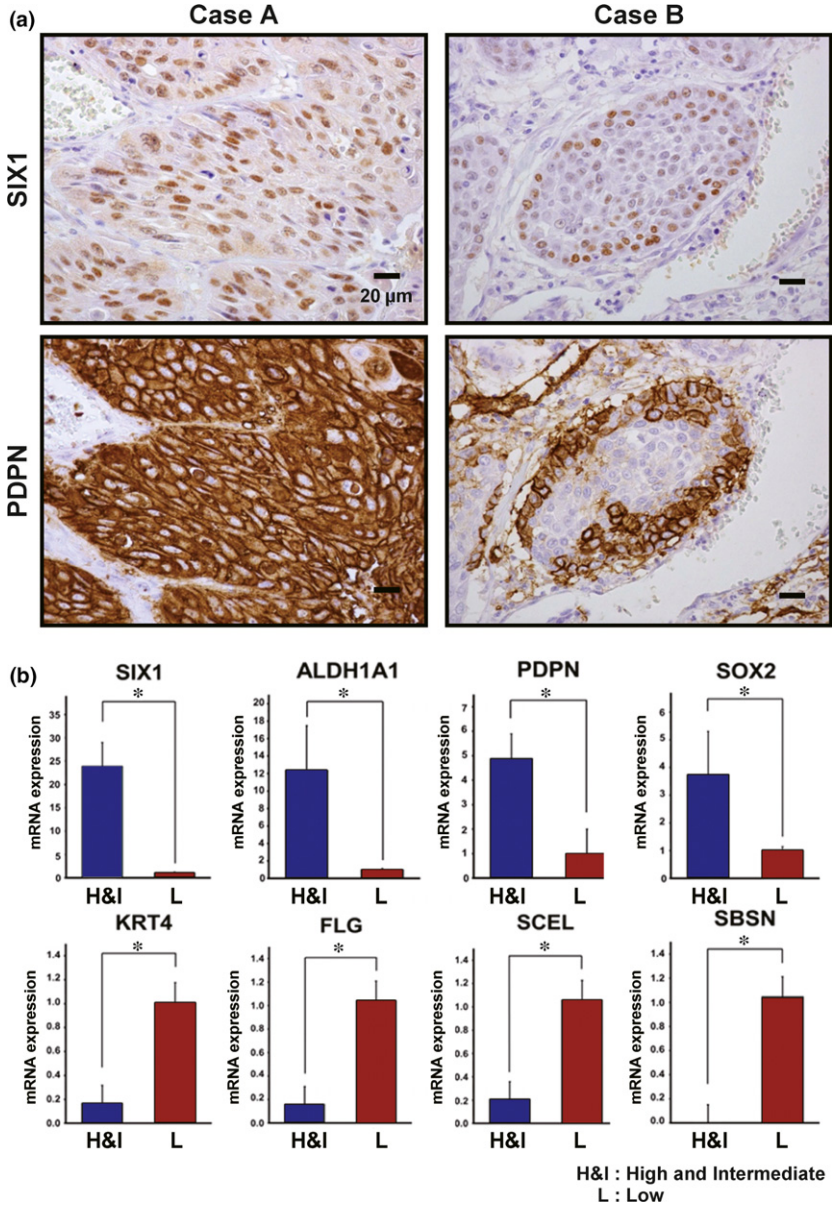

Fig. 5. Predominant expression of SIX1 in locally advanced esophageal squamous cell carcinoma (ESCC) patients with high expression of basal cell markers. (a) Immunohistochemistry of PDPN and SIX1 in serial sections obtained from two ESCCs. (b) Quantitative RT-PCR of *SIX1*, basal cell markers (*ALDH1A1*, *PDPN*, and *SOX2*), and differentiated cell markers (*KRT4*, *FLG*, *SCEL*, and *SBSN*) in 85 biopsy samples from locally advanced ESCC patients before definitive CRT. H&I: high (40 cases) and intermediate (25 cases) in *SIX1* mRNA level, L: low (20 cases) in *SIX1* mRNA level. Data are mean ± SD from three independent experiments. * $P < 0.05$.

histopathological analysis revealed this tumor tissue to be composed of PDPN-expressed cells and showed poorly differentiated histology (Fig. 4e). These results suggest that SIX1 enhances tumorigenicity and invasiveness and maintains or increases PDPN-positive cancer stem cells.

Correlation between expression of SIX1 and tumor basal cell markers in poor prognostic ESCC patients. We investigated colocalization of PDPN and SIX1 in tumor basal cells by serial section. In case A, both SIX1 and PDPN are diffusely stained, consistent with xenografted tumors of poorly-differentiated transfectant TE6-SIX1-17 (Figs 4e, 5a). In case B, they are colocalized at the peripheral zone where the tumor basal cells existed in the well-differentiated type of ESCCs, consistent with xenografted tumors of well-differentiated cell line KYSE510 (Figs 5a and S7). We further examined the correlation between mRNA expression of *SIX1* and that of basal cell markers or differentiated markers from 85 locally advanced ESCC patients. In the *SIX1* high and intermediate cases (40 and 25 cases, respectively), all basal cell markers (*PDPN*, *ALDH1A1*, and *SOX2*) were highly expressed compared with the *SIX1* low cases (20 cases) (Fig. 5b). In contrast, well-known differentiation markers of squamous cells (*KRT4*, *FLG*, *SCEL*, and *SBSN*) were significantly suppressed in the *SIX1* high cases (Fig. 5b). These results also support the notion that SIX1 promotes cancer stem cell self-renewal and suppresses differentiation. Although transient transfection of *SIX1* cDNA or siRNA did not alter PDPN expression (data not shown), these results indicate that SIX1 cannot directly induce *PDPN* expression, but can accelerate self-renewal of PDPN-positive tumor basal cells both *in vitro* (Fig. 4d) and *in vivo* (Fig. 4e).

To further investigate the correlation between SIX1 and PDPN in ESCC patients received by chemoradiotherapy followed by surgery, immunohistochemical analyses of some normal tissues and 85 ESCCs were performed. Although *PDPN* mRNA was detected in both the basal and epibasal cell layers (Figs 1a, 4a), PDPN protein was stained only at the basal cells in normal esophagus (Fig. 6a). Therefore, the expression of PDPN is independent from that of SIX1 in the normal epithelium. In primary ESCCs, the PDPN distribution differed among cases (Fig. 6a). Positive type 1 case was diffusely and strongly stained by anti-PDPN antibody. In positive type 2 case, PDPN staining was not strong but diffuse. Although the staining appeared weak in the positive type 3 case because of abundant stroma, almost all the cancer cells were stained. Positive types 1, 2, and 3 showed poorly-differentiated histology and were determined as totally positive, whereas positive type 4 case showed a well-differentiated histology and was stained only at the periphery of the tumor nest. Notably, PDPN-negative cases (43/85, 51%) or positive type 4 cases (26/85, 30%) showed a significantly better prognosis than those with positive types 1–3 cases (16/85, 19%) (Fig. 6b).

Blockade of TGF-β signaling diminishes PDPN-positive cancer stem cells. Finally, we tried blocking of TGF-β signaling to examine whether self-renewal of ESCC cells is dependent on

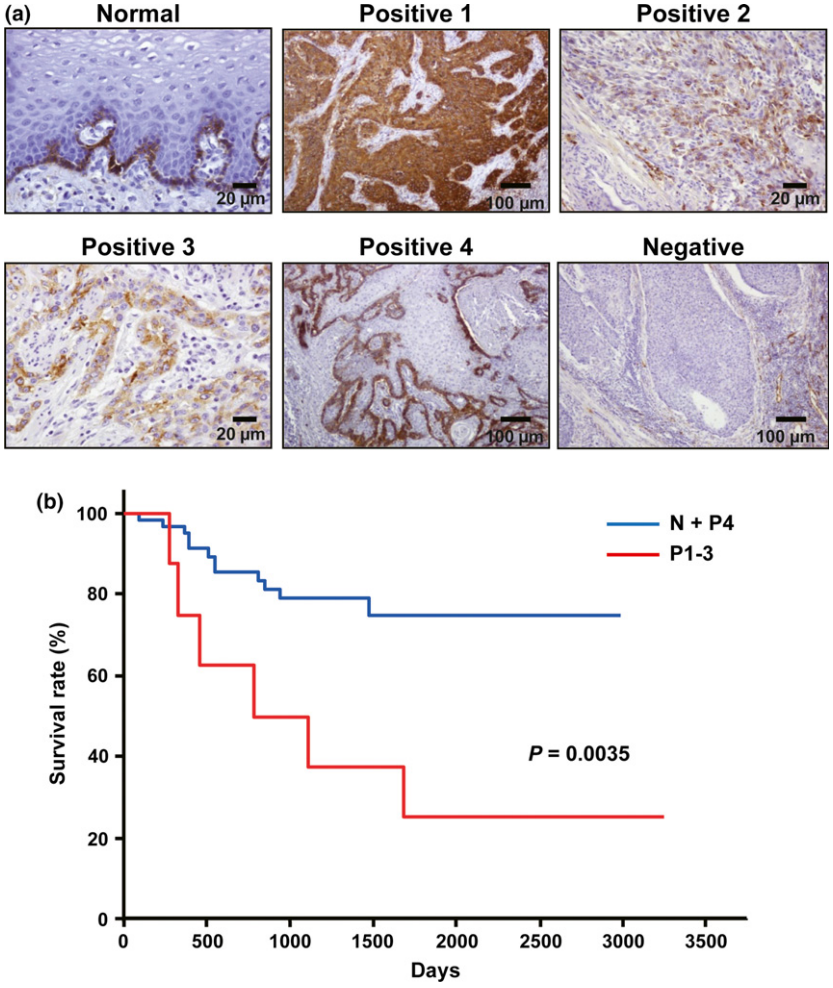

Fig. 6. PDPN co-localized with SIX1 is a poor prognostic factor in esophageal squamous cell carcinoma (ESCC). (a) Representative patterns of immunohistochemical staining of PDPN in normal esophagus and primary ESCCs. Section is counterstained with hematoxylin. (b) Overall survival of 85 ESCC patients classified by PDPN staining patterns. P1-3: 16 cases with three positive staining patterns (positive 1-3), N+P4: 69 cases with a positive staining pattern, positive 4 (n = 26) or negative (n = 43). P-values are calculated by log-rank test.

this signal pathway. The PDPN-positive cell population in 8 ESCC cell lines (TE1, TE3, TE5, TE6, TE8, TE10, T.Tn, and KYSE510) was 0.1, 85.3, 24.3, 3.4, 90.2, 1.0, 88.3, and 25.8%, respectively. After exposure to small molecular inhibitor of TGF-β receptor A-83-01[23] for 7 days, the relative viability was significantly decreased in all of the four ESCC cell lines (TE3, TE8, T.Tn, and KYSE510) harboring a high PDPN-positive cell population (Fig. 7a). In TE3 and KYSE510 highly sensitive to A-83-01, its PDPN-positive cell population was significantly reduced by A-83-01(Fig. 7b). Conversely, TGF-β treatment increased PDPN-positive cell population in TE3 and KYSE510 (Fig. S8).

The status of PDPN overexpression in two transfectants, TE6-SIX1-13 and TE6-SIX1-17, was examined by Western blotting (Fig. S9). Unfortunately, PDPN expression in TE6-SIX1-13 was decreased by multiple passages for a year. Therefore, we used only TE6-SIX1-17 in these experiments. We first showed that the phospho-Smad2/3 level increases more in TE6-SIX1-17 than in TE6-Mock after TGF-β treatment in a dose dependent manner, suggesting that TGF-β signal is activated by SIX1 (Fig. 7c, upper), and also confirmed that the phosphorylation of Smad2/3 was inhibited by A-83-01 (Fig. 7c, lower). Accordingly, the relative viability was significantly low in TE6-SIX1-17 compared with TE6-Mock (Fig. 7d). PDPN-positive cell population was also reduced in TE6-SIX1-17 but not in TE6-Mock. Representative FACS data were shown (Fig. 7e).

Discussion

The Six family of homeodomain transcription factors plays an important role in early embryogenesis[9] and normal development.[10–12] Among this family, *SIX1* has been reported to express aberrantly in various types of tumors including hepatocellular carcinoma,[13] cervical cancer,[14] ovarian cancer,[15] colorectal cancer,[16] and breast cancer.[18] *SIX1* has also been reported as being responsible for tumor progression, invasion or metastasis in some cancers such as hepatocellular carcinoma,[24] rhabomyosarcoma,[25] and breast cancer.[18] In infiltrating ductal breast carcinomas, the aberrant expression of *SIX1* was caused predominantly by gene amplification.[26] Despite the strong relation between *SIX* expression and cancer progression that has been reported, detailed molecular functions of the *SIX1* as an oncogene are at the beginning of understanding. For instance, CCNA1 has been reported to be a downstream effector of SIX.[27] EZR has been shown to be a target of SIX1 and to activate several oncogenes.[25] SIX1 inhibits TRAIL-mediated apoptosis in ovarian cancer,[15] and

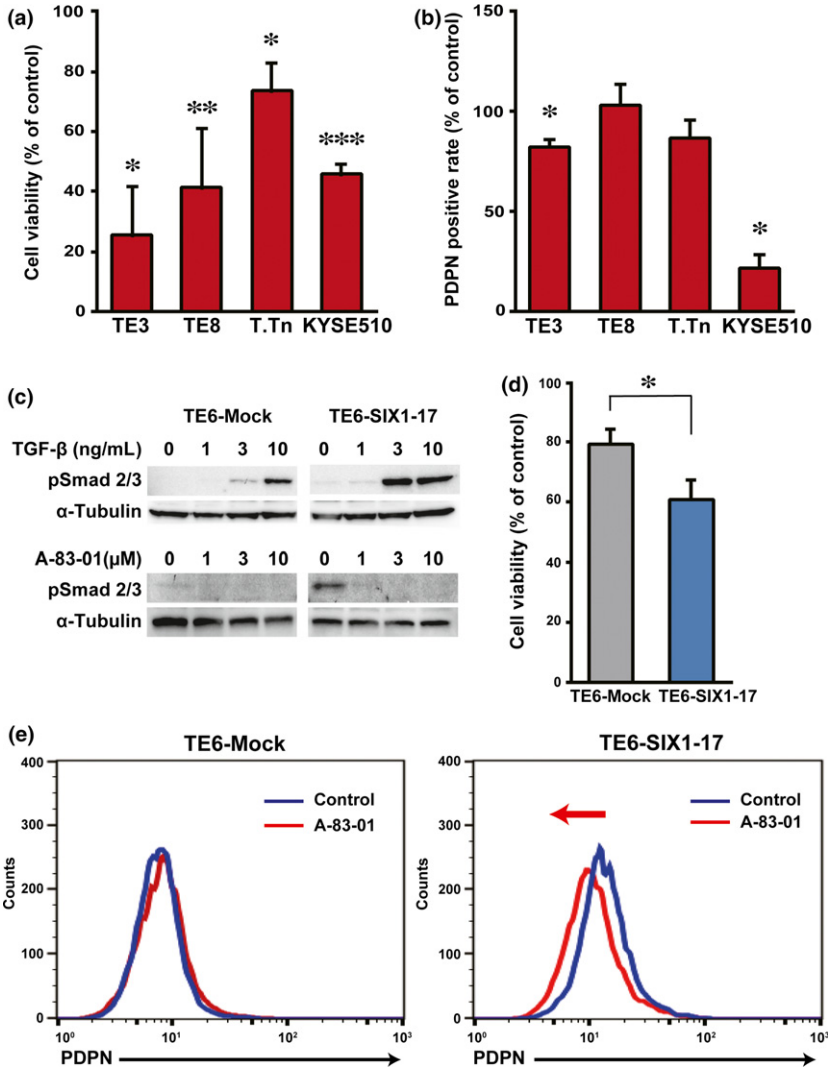

Fig. 7. Blockade of transforming growth factor-β (TGF-β) signaling induces cell death and reduces PDPN-positive cell population. (a, b) Cell viability and PDPN-positive rate of 4 ESCC cell lines (TE3, TE8, T.Tn., and KYSE510) with a high PDPN cell population at 7 days exposure by 10 μM TGF-β receptor inhibitor (A-83-01). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$. (c) Induction of the phospho-Smad2/3 at 24 h after TGF-β treatment and reduction of the phospho-Smad2/3 at 48 h after A-83-01 treatment in the nuclei of TE-Mock and TE6-SIX1-17. (d, e) Cell viability and PDPN-positive cell population of TE6-Mock and TE6-SIX1-17 at 7 days exposure by 10 μM A-83-01. Data are mean ± SD from three independent experiments. * $P < 0.05$.

induces EMT through ZEB1 activation and miR200 repression in colorectal cancer.[16] In breast cancer, Micallizi *et al.* and Farabaugh *et al.* have reported that SIX1 also mediates EMT and induces cancer stem cell phenotypes through TGF-β signaling.[19,20] Another study has reported that SIX1 switches TGF-β signaling from tumor suppressive to tumor promoting by regulating miR106b-25 and Smad7.[28] In ESCC, we found that *SIX1* is also aberrantly expressed (Figs 1b, 2). More significantly, the cases with high *SIX1* expression showed high lymph node metastasis and poor survival in surgery alone or chemoradiotherapy followed by surgery (Figs 1c,d, 6b). These findings strongly indicate that SIX1 is involved in cancer malignancy by promotion of metastasis. On the contrary, in ESCC patients who received definitive CRT, no significant difference is found, though cases with high SIX1 expression had a tendency for a worse prognosis (data not shown). This is partly because the malignant potential cannot be attributed to only one transcription factor. Many factors are reported to be associated with EMT in ESCC such as TWIST1,[29] SNAI1,[30] hedgehog pathway,[31] and FOXA1.[32]

Transient transfection studies of *SIX1* cDNA and siRNA showed that SIX1 induced EMT and invasiveness in ESCC (Fig. 3a,b), and the stable *SIX1*-transfectants showed high invasiveness without EMT (Figs 4c and S5). It should be noted that the reduction of *CDH1* mRNA and the induction of an EMT marker (*VIM*) and five EMT regulators by SIX1 were not observed in the two SIX1-transfectants (Fig. S5). These data suggested that the role of SIX1 in ESCC is the induction of TGF-β signal-related genes but is unclear in the authentic EMT induction.

In accordance with previous findings in breast cancers, SIX1 activated TGF-β (Fig. 3a,d). Furthermore, we investigated whether SIX1 induces a cancer stem cell phenotype, since EMT is often accompanied by cancer stem cell properties.[5] We first found that SIX1 induces some genes selectively expressed at the normal basal layer where esophageal tissue stem cells are thought to reside (Fig. 4a,b). Although little is known about the cancer stem cells of ESCC,[6] PDPN has been reported to be a cancer stem cell marker of oral squamous cell carcinoma.[21] In addition, Rahadiani *et al.* reported PDPN-positive ESCC cells have a tumor initiating ability.[22] We confirmed that PDPN protein expression is selectively expressed at tumor basal cells of xenografted tumors and primary ESCCs (Figs 6a and S7). Thus, we used PDPN as a cancer stem cell marker of ESCC. Both *SIX1*-overexpressing stable cell lines (TE6-SIX1-13 and TE6-SIX1-17) had a higher population of PDPN-positive cells compared to that of TE6-Mock (Fig. 4d). In addition, PDPN expression was still high in the xenografted tumor of a SIX1-stably expressing ESCC cell line (Fig. 4e). Therefore, these studies indicate that consistent *SIX1* overexpression accelerated self-renewal of PDPN-positive cancer stem cells and promoted tumor progression. Co-expression between *SIX1* and three basal cell markers (*PDPN*, *ALDH1A1*, and *SOX2*) and reverse correlation between *SIX1* and four differentiation markers (*KRT4*, *FLG*, *SCEL*, and *SBSN*) in biopsy samples of locally advanced ESCC patients supported this notion (Fig. 5b).

Although TGF-β signaling induces EMT in various tumors,[33,34] this mechanism is not well-known in ESCC. Recently, Natsuizaka *et al.* reported that immortalized esophageal epithelial cells underwent EMT through TGF-β.[35] Our data showed that *TGFB* was upregulated by *SIX1* (Fig. 3a,d). It is reported that the autocrine of TGF-β mediates tumorigenicity through SOX2/SOX4 in glioblastoma.[36] Likewise, we found SOX2/SOX4 is upregulated as well as TGFB by *SIX1* transfection (Fig. 3d). In addition, treatment by A-83-01, a small-molecule inhibitor of the TGF-β receptor, suppressed growth of SIX1- and PDPN-expressing ESCC cells (Fig. 7d,e). This result indicates that self-renewal of cancer stem cells of ESCC promoted by SIX1 is dependent on TGF-β signaling. To our knowledge, this is the first report that shows the effectiveness of the TGF-β receptor inhibitor for ESCC. More significantly, there is no finding about a specific inhibitor of the cancer stem cell of ESCC.

In conclusion, our study demonstrated that aberrant expression of SIX1 is correlated with metastasis and poor survival in ESCC patients. We also found that SIX1 accelerates self-renewal of cancer stem cells of ESCCs. This process is dependent on TGF-β signaling, and TGF-β receptor inhibitors may be effective in highly malignant ESCC with high SIX1 expression.

Acknowledgements

This work was supported by Japan Agency for Medical Research and Development (Practical Research for Innovative Cancer Control), National Cancer Center Research and Development Fund, The Ministry of Education, Culture, Sports, Science and Technology of Japan, and The Princess Takamatsu Cancer Research Fund. Drs T Nishimura, M Tamaoki, M Komatsu were the recipients of a Research Resident Fellowship from the Foundation of Promotion of Cancer Research in Japan. We thank Drs. Kanako Nakamura and Tomoko Hiroki for excellent technical advice. This work was carried out with the kind cooperation of the Core Facility of the National Cancer Center Research Institute.

Disclosure Statement

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Relative migration ability of eight ESCC cell lines (TE8, TE3, T.Tn, KYSE510, TE1, TE5, TE6, and TE10) and an oral cancer cell line (A431) assessed by matrigel invasion assay.

Fig. S2. Quantitative RT-PCR of TGF-β signal-related genes (*TGFB2*, *TGFBI, TGFBR1, TGFBR2*, *SOX2, and SOX4*) in TE3 with *SIX1* siRNA treatment. * $P < 0.05$.

Fig. S3. Photographs of 5 full uncut gels before cropping to make Fig. 3d were shown.

Fig. S4. Expression of *SIX1* and TGF-β signal-related genes (*TGFB2*, *TGFBI, TGFBR1, TGFBR2*, and *TGFBR3*) in the parental TE6 cell line (TE6-Parent) and its mock-transfectants (TE6-Mock).

Fig. S5. Expression of an epithelial cell marker (CDH1), an EMT marker (VIM) and 5 EMT regulators (SNAI2, ZEB1, ZEB2, TWIST1, and TWIST2) in the three SIX1-transfectants (TE6-Mock, TE6-SIX1-13, and TE6-SIX1-17).

Fig. S6. Photographs of 4 full uncut gels before cropping to make Fig. 4b were shown.

Fig. S7. Representative images of HE staining and immunohistochemistry for PDPN in xenografted tumors of a well-differentiated ESCC cell line, KYSE510.

Fig. S8. TGF-β treatment increased PDPN-positive cell population in TE3 and KYSE510. * $P < 0.05$, ** $P < 0.01$.

Fig. S9. Western blots to evaluate the stability of SIX1 expression in two transfectants after multiple passages. SIX1 (left), ACTB (right).

Table S1. Primer sequences for RT-PCR.