SOX2-silenced squamous cell carcinoma: a highly malignant form of esophageal cancer with SOX2 promoter hypermethylation

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This study originally aimed to investigate whether the overexpression of SOX2 is associated with the poor prognosis of patients with squamous cell carcinoma of the esophagus. However, we unexpectedly found that esophageal squamous cell carcinomas completely lacking SOX2 expression showed distinct pathologic features and highly aggressive clinical courses. The study cohort consisted of 113 consecutive patients with esophageal squamous cell carcinoma who underwent surgical resection without neoadjuvant therapy. Immunostaining on tissue microarrays and whole sections revealed that 8/113 (7%) cases were entirely negative for this transcriptional factor. SOX2-negative cancers were histologically less differentiated ($P=0.002$) and showed higher pT and pStages ($P=0.003$ and 0.007 , respectively) than SOX2-positive cases. A remarkable finding was widespread lymphatic infiltration distant from the primary invasive focus, which was observed in 4 SOX2-negative cancers (50%), but none of the SOX2-positive cases. All separate dysplastic lesions observed in SOX2-negative cases were also SOX2-negative. The negative expression of SOX2 appeared to be an independent poor prognostic factor (OR = 7.05, 95% CI = 1.27–39.0). No mutations were identified in the coding or non-coding regions of SOX2. Fluorescent *in situ* hybridization did not show any copy-number variations in this gene. Since the SOX2 promoter contains an extensive CpG island, SOX2-negative cases underwent methylation-specific PCR, which disclosed promoter hypermethylation in all cases. In conclusion, SOX2-silenced squamous cell carcinomas of the esophagus appear to be a minor, but distinct form of malignancy characterized by extensive lymphatic invasion, a poor prognosis, and potential association with multiple SOX2-negative neoplastic lesions. The hypermethylation of the promoter region is seemingly a critical epigenetic event leading to SOX2 silencing.

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Esophageal cancer is the sixth leading cause of cancer death and eighth most frequently diagnosed malignancy.¹ Approximately 90% of esophageal cancers diagnosed worldwide are histologically squamous cell carcinomas.¹ Despite recent advances in therapeutic modalities and treatment options, the 5-year survival rate of esophageal squamous cell

carcinomas remains less than 15%.^{1,2} Early esophageal cancers are typically asymptomatic and, thus, difficult to diagnose. Another factor causing a poor prognosis is that distant or nodal metastases may develop, even in cases in which primary tumors are restricted to the mucosal or submucosal tissue.^{2,3} Although molecular testing for gene fusions and amplifications has increasingly been used for other malignancies such as lung and breast cancers, marker-based stratification schemes are not currently available for squamous cell carcinomas of the esophagus.²

SOX2, a SRY-related HMG-box transcription factor, has a critical role in the self-renewal and

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pluripotency of embryonic stem cells.⁴ The oncogenic role of *SOX2* was initially described in 2009.⁵ A peak in genomic amplification on chromosome 3q26.33 containing *SOX2* was found in 6/30 (15%) cases of esophageal squamous cell carcinomas. A fluorescence *in situ* hybridization analysis on tissue samples of an independent cohort of esophageal squamous cell carcinomas identified *SOX2* amplification in 7/63 (11%) patients.⁵ *In vitro* studies also revealed that *SOX2* expression was essential for the proliferation of *SOX2*-overexpressing cell lines.⁵ Furthermore, the ectopic expression of *SOX2* cooperated with *FOXE1* or *FGFR2* transformed immortalized tracheobronchial epithelial cells.⁵ Based on these findings, *SOX2* has been increasingly recognized as an oncogene and potential drug target in squamous cell carcinomas of the esophagus.⁶ The amplification and overexpression of *SOX2* were also demonstrated in squamous cell carcinomas of other organs (eg, the lungs, larynx, pharynx, uterine cervix, penis, and skin).⁷

The gene amplification and immunohistochemical expression of *SOX2* are known to be moderately, but not entirely correlated.⁸ This study was performed based on our original hypothesis that the immunohistochemical overexpression of *SOX2* may be associated with a poor prognosis in patients with esophageal squamous cell carcinoma. However, we unexpectedly found that esophageal cancers completely lacking *SOX2* expression showed highly aggressive clinical courses. Their clinicopathologic features appeared to be distinct from those of *SOX2*-retained tumors. The underlying molecular mechanisms of *SOX2* silencing were also investigated by gene sequencing, fluorescent *in situ* hybridization, and methylation assays of the promoter region.

Materials and methods

Case Selection and Tissue Samples

This study was approved by the Ethics Committee of Kobe University Graduate School of Medicine. The study cohort consisted of 113 consecutive patients with esophageal squamous cell carcinoma who underwent surgical resection without neoadjuvant therapy at Kobe University Hospital between November 2005 and December 2015. Patients who received preoperative therapies before surgery were excluded because chemotherapy or radiotherapy may bias the evaluation of *SOX2* expression. A possible example is that *SOX2*-positive cancers may become *SOX2*-negative after neoadjuvant therapy due to the cancer cells expressing *SOX2* being eliminated. According to the protocol in our institute, the resected esophagus was entirely submitted for histologic examination in order to identify separate dysplastic lesions. Formalin-fixed paraffin-embedded tissue was used for pathologic analyses and molecular studies.

Clinical information including patient prognoses was obtained from stored medical records and imaging. All patients were followed-up every 6 months after surgery. The median follow-up period until the time of writing this manuscript was 52 months (range 2–125). Histology slides were also reviewed in terms of the depth of invasion, degree of differentiation, lymphovascular invasion, and the presence or absence of multiple lesions. Lymphatic or venous invasion was evaluated on D2-40- or elastic van Gieson-stained sections, respectively. Tumor stages were defined based on the TNM classification system (UICC 7th edition⁹).

Immunohistochemistry

Tissue microarray blocks were constructed using Tissue Microarrayer JF-3 (Pathology Institute, Toyama, Japan). Three representative tissue cores (2 mm in diameter) were obtained from each case. In addition to *SOX2*, CD133 and p63 were also immunostained in order to determine how the expression of *SOX2* is correlated with the expression of other stem/basal cell markers.

Immunostaining for *SOX2*, CD133, p63, and D2-40 was performed on a Bond Max autostainer (Leica Microsystems, Wetzlar, Germany) according to the manufacturers' protocols. Deparaffinized sections were heat-treated using pH9.0 ER2 solution (Leica Microsystems) for 10 min and incubated with primary antibodies. The antibodies used were a rabbit monoclonal antibody against human *SOX2* (clone SP76, dilution 1:100, Abcam, Cambridge, UK), mouse monoclonal antibody against human CD133 (clone 80B258, 5 µg/ml, made in one of our laboratories¹⁰), mouse monoclonal antibody against p63 (clone 4A4, pre-diluted, Nichirei Bioscience, Tokyo, Japan), and mouse monoclonal antibody against D2-40 (clone D2-40, dilution 1/50, Dako Cytomation, Glostrup, Denmark). Positive controls were the normal prostate (*SOX2*), pancreas (CD133), and skin (p63 and D2-40) according to datasheets of antibodies and previous reports.¹⁰

Expression levels of *SOX2*, CD133, and p63 were evaluated semi-quantitatively according to the percentage of positive cells: negative (no expression); focal (1–25%); moderate (26–50%); extensive (>50%). When all three cores were negative for *SOX2*, a whole section of cancer tissue was also stained in order to ensure that the expression of *SOX2* was entirely absent in other regions. In patients with multiple esophageal cancers, the largest cancer was selected for tissue microarrays. When the largest tumor was *SOX2*-negative, the expression of *SOX2* in all other lesions including intraepithelial neoplasia was also examined. Furthermore, in *SOX2*-negative cases, all metastatic deposits in lymph nodes dissected at the initial surgery and recurrent tumors biopsied during the follow-up were stained for *SOX2* in order to eluci-

date whether the expression of this transcriptional factor emerges during tumor progression.

Gene Sequencing

The DNA sequencing of *SOX2* was performed in all *SOX2*-negative cases. Genomic DNA was extracted from formalin-fixed paraffin-embedded tissue blocks. Areas consisting of a large proportion of tumor cells (>30%) were selected for DNA extraction under a microscope. DNA extraction was performed using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA content was assessed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

SOX2 contains a single exon (Gene ID 6657). Four sets of PCR primers were prepared to cover the entire exon. The promoter and 5'/3'-untranslated regions were also amplified using 3 and 8 sets of primers, respectively. Primer sequences are described in Supplementary Table 1. PCR was performed using 10–40 ng of genomic DNA, 10 pmol each of the forward and reverse primers (Hokkaido System Science, Hokkaido, Japan), and 15 μ l of GoTaq Green Master Mix 2x (Promega, Madison, WI, USA) in a total volume of 30 μ l. The PCR thermocycling conditions used were as follows: 95 °C for 1 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min. Amplification products were purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega). Cycle sequencing was performed using BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA, USA) and the amplified products were separated by capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Fluorescent *In Situ* Hybridization

Fluorescent *in situ* hybridization for *SOX2* was performed in all *SOX2*-negative cases in order to elucidate whether *SOX2*-negative tumors have copy-number losses. The *SOX2* target probe spanning the locus 3q26.33 (BAC clone CTD-2348H10, Invitrogen, Carlsbad, CA, USA) and a commercially available centromeric probe on chromosome 3 (Agilent Technologies, Santa Clara, CA, USA) were selected for hybridization. The copy-number per cell was enumerated on at least 50 tumor cells.

Assessment of *SOX2* Promoter Methylation

The methylation status of the *SOX2* promoter was analyzed as a potential epigenetic down-regulation mechanism of gene expression. Similar to other key developmental genes, which have high CpG density promoters, *SOX2* contains an extensive CpG island before the transcription start site.¹¹ Primer sequences

for methylation-specific PCR were obtained from a previous study (Supplementary Table 1).¹²

The bisulfite modification of genomic DNA was performed using the EZ DNA methylation kit (Zymo Research, Irvine, CA, USA). In this process, unmethylated cytosine residues in genomic DNA are converted to uracil by sodium bisulfite. Methylation-specific PCR was performed using Takara EpiTaq HS (for bisulfite-treated DNA; Takara Bio, Shiga, Japan). The PCR thermocycling conditions used were as follows: 98 °C for 1 min, followed by 50 cycles of 98 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s. Reaction products were visualized by electrophoresis with ethidium bromide. The density of the bands was analyzed using ImageJ (version 1.41, National Institute of Health, Maryland) for semi-quantification. The ratio of the density of methylated alleles to that of unmethylated ones was calculated for each sample.

Statistical Analysis

Continuous and categorical variables in each group were analyzed using an unpaired *t*-test, chi-squared test, and Fisher's exact test. The overall survival, disease-specific survival, and recurrence-free survival of patients were compared between *SOX2*-positive and -negative cases using the Kaplan–Meier method, and differences between survival curves were evaluated with the Log-rank test. A multivariate analysis was also performed on prognostic factors assessed by Cox's proportional hazards model. A probability of $P < 0.05$ was considered to be significant. The software used for statistical analyses was JMP ver.13 software (SAS Institute Inc, North Carolina, USA).

Results

SOX2 Expression in Squamous Cell Carcinomas of the Esophagus

SOX2 expression was retained in 105/113 (93%) esophageal squamous cell carcinomas. Its expression was focal (1–25% of cancer cells) in 3 cases, moderate (26–50%) in 14 cases, and extensive (>50% of cells) in 88 cases (Figure 1). The remaining 8 (7%) appeared to be negative for this transcriptional factor in the tissue microarray study, and the complete loss of expression was further confirmed by additional staining on whole sections (Figure 1).

In *SOX2*-negative cases, the expression of this stemness molecule was completely lost even in intraepithelial areas, which was in clear contrast to the adjacent non-neoplastic epithelium, in which *SOX2* was constantly expressed particularly in the lower 1/4 to 3/4 of the layer. Three out of eight *SOX2*-negative cases had intraepithelial neoplasia elsewhere of the esophagus (8 lesions; 1 to 6 lesions per case). Interestingly, all lesions were also

Figure 1 SOX2 immunostaining in squamous cell carcinomas of the esophagus. (a) SOX2 is diffusely expressed in cancer cells (a microarray section, $\times 200$). (b) No expression of SOX2 is observed (a microarray section, $\times 200$). (c) Although non-neoplastic esophageal glands (np) are positive for SOX2, cancer cells lack the expression of this transcriptional factor (a whole section, $\times 100$). (d) The lack of SOX2 expression in intraepithelial cancer (arrows) is in contrast to that in the SOX2-positive non-neoplastic epithelium (np).

Figure 2 Complete loss of SOX2 expression in high-grade intraepithelial neoplasia observed in a case of SOX2-negative esophageal squamous cell carcinoma (patient 7). Although the non-neoplastic squamous epithelium (np) is positive for SOX2, high-grade intraepithelial neoplasia lacks its expression. SOX2 immunostaining, $\times 100$.

completely SOX2 negative (Figure 2). In contrast, randomly selected separate dysplastic lesions ($n = 10$) in SOX2-positive cases expressed this transcriptional factor to variable degrees. SOX2 negativity was also confirmed in metastatic deposits in regional lymph nodes ($n = 38$) removed at the time of initial surgery and a recurrent tumor ($n = 1$) biopsied during the follow-up in SOX2-negative cases.

Clinicopathologic Features

The pathologic features of SOX2-negative esophageal cancers are summarized in Table 1. SOX2-positive ($n = 105$) and -negative cases ($n = 8$) are compared hereafter. As shown in Table 2, no significant difference was observed between the two groups in terms of clinical features including age, gender, smoking and drinking histories, and serum tumor markers. Regarding pathologic parameters, SOX2-negative esophageal cancers were histologically less differentiated ($P = 0.002$) and

Table 1 Pathologic features of SOX2-negative squamous cell carcinomas of the esophagus

	Age/ Gender	Size	Gross appearance	Histology	Lymphatic infiltration	Multiple lesions	Stage
Pt1	57 M	117 mm	Flat	Poorly differentiated squamous cell carcinoma consisting of cancer cells arranged in nests showing extensive intraepithelial spread. Widespread lymphatic infiltration is observed in almost the entire esophagus and gastric fundus. In addition to direct tumor invasion down to the submucosal layer, marked lymphatic infiltration is noted in the muscularis propria and adventitia (Figure 3).	Extensive	None	pT3N1M0
Pt2	62 M	110 mm	Flat	Moderately differentiated squamous cell carcinoma with extensive intraepithelial spread. Although direct invasion is restricted to the lamina propria, widespread lymphatic infiltration is observed throughout the esophageal wall.	Extensive	Low-grade intraepithelial neoplasia ($n = 1$)	pT3N1M0
Pt3	60 M	80 mm	Ulcerative	Poorly differentiated squamous cell carcinoma with tumor cells arranged in a solid nested pattern. Lymphatic infiltration is observed in an area distant from the tumor mass.	Extensive	None	pT3N1M0
Pt4	67 M	50 mm	Ulcerative	A localized tumor consisting of moderately differentiated squamous cell carcinoma. Cancer cells are arranged in a solid architecture. Several foci of lymphatic infiltration are observed in remote areas.	Extensive	High-grade intraepithelial neoplasia ($n = 1$)	pT2N1M0
Pt5	73 M	18 mm	Slightly elevated	Poorly differentiated squamous cell carcinoma with cancer cells arranged in solid sheets. Multiple foci of lymphatic invasion are present, particularly at the invasive front.	Moderate	None	pT1N0M0
Pt6	84 F	26 mm	Ulcerative	Poorly differentiated squamous cell carcinoma consisting of tumor cells arranged in a solid pattern with foci of borderline spindle cell changes. Lymphatic invasion is present mainly inside the tumor mass.	Moderate	None	pT1N0M0
Pt7	61 F	20 mm	Nodular	Poorly differentiated squamous cell carcinoma with cancer cells arranged in an irregular trabecular architecture shows scirrhous infiltration.	Focal	High-grade intraepithelial neoplasia ($n = 6$)	pT2N1M0
Pt8	72 M	85 mm	Ulcerative	Moderately differentiated squamous cell carcinoma consisting of sheeted tumor cells with occasional foci of keratinization.	Focal	None	pT3N1M0

Table 2 Comparison of clinical features between SOX2-negative and -positive groups

	SOX2 negative (n = 8)	SOX2 positive (n = 105)	P-value
<i>Age</i>			
Median	65	66	0.685
Range	(57–84)	(45–80)	
<i>Gender</i>			
Male	6 (75%)	89 (85%)	0.611
Female	2 (25%)	16 (15%)	
<i>Smoking</i>			
Non-smoker	2 (25%)	17 (16%)	0.658
Smoker	6 (75%)	78 (74%)	
Unknown	0	10 (9%)	
<i>Drinking</i>			
Non-drinker	1 (13%)	6 (7%)	0.655
Drinker	7 (87%)	89 (84%)	
Unknown	0	10 (9%)	
<i>Multiple lesions</i>			
Present	3 (38%)	34 (33%)	0.926
Absent	5 (62%)	71 (67%)	
<i>Tumor marker SCC</i>			
≥ 1.5 ng/ml	1 (13%)	36 (34%)	0.269
< 1.5 ng/ml	6 (74%)	64 (61%)	
Not measured	1 (13%)	5 (5%)	
<i>Tumor marker CEA</i>			
≥ 5.0 ng/ml	0	15 (14%)	0.549
< 5.0 ng/ml	7 (87%)	82 (78%)	
Not measured	1 (13%)	8 (8%)	

showed more advanced pT and pStages ($P=0.003$ and 0.007 , respectively) than SOX2-positive cases (Table 3). A remarkable histologic finding in SOX2-negative cases was extensive lymphatic invasion. Widespread lymphatic infiltration distant from the primary invasive focus was observed in 4/8 (50%) SOX2-negative cancers, but in none of the SOX2-positive cases (Figure 3). In the remaining cases, lymphatic infiltration was focal (< 5 foci per section) or moderate (5–10 foci) (Table 1).

The relationship between SOX2 and CD133 (prominin-1) expression was examined in order to elucidate how the expression of these two stem cell markers are related. As shown in Table 4, although 7/8 (88%) SOX2-negative cases lacked CD133 expression, 78/105 (74%) SOX2-positive cancers were also negative for CD133 with no correlation between the two groups. No correlation was further observed between SOX2 and p63 (a marker for basal and para-basal cells) (Table 4).

Post-Operative Outcome

During the follow-up, tumor recurrence and cancer-related death were more frequently observed in

Table 3 Comparison of pathologic features between SOX2-negative and -positive groups

	SOX2 negative (n = 8)	SOX2 positive (n = 105)	P-value
<i>Tumor size</i>			
Median	65 mm	33 mm	0.270
Range	18–117 mm	5–140 mm	
<i>Location</i>			
Cervical	1 (13%)	4 (4%)	0.016
Thoracic	5 (62%)	98 (93%)	
Abdominal	0	3 (3%)	
Diffuse	2 (25%)	0	
<i>Degree of differentiation</i>			
Well to moderately	3 (38%)	101 (89%)	0.002
Poorly	5 (62%)	12 (11%)	
<i>Vascular invasion</i>			
Present	6 (75%)	31 (30%)	0.014
Absent	2 (25%)	74 (70%)	
<i>Lymphatic invasion</i>			
Present	8 (100%)	50 (48%)	< 0.001
Absent	0	55 (52%)	
<i>pT classification</i>			
pT1	2 (25%)	82 (78%)	0.003
pT2–4	6 (75%)	23 (22%)	
<i>Lymph node metastasis</i>			
Present	6 (75%)	70 (67%)	0.026
Absent	2 (25%)	35 (33%)	
<i>pStage</i>			
pStage I	2 (25%)	64 (61%)	0.007
pStages II–IV	6 (75%)	41 (39%)	
<i>Recurrence</i>			
Present	5 (62%)	18 (17%)	0.009
Absent	3 (38%)	87 (83%)	
Disease-specific mortality	4 (50%)	8 (8%)	0.004

SOX2-negative cases than in SOX2-positive cancers ($P=0.009$ and 0.004 , respectively) (Table 2). Five patients developed recurrence by the time of writing this manuscript in the abdominal lymph nodes ($n=5$), parabrachial lymph nodes ($n=1$), an anastomotic site ($n=1$), the liver ($n=1$), bone ($n=1$), and/or duodenum ($n=1$). The recurrent tumor in the duodenum presented with a slightly elevated mucosal lesion, a biopsy from which showed cancer cells in dilated lymphatics of the lamina propria, but no infiltration outside the vessels (Figure 4).

Recurrence-free and disease-specific survival were significantly shorter in patients with SOX2-negative esophageal cancer than in patients with SOX2-positive malignancy (both $P<0.001$) (Figure 5). The median recurrence-free survival was 11 months for SOX2-negative cases and 51 months for the SOX2-positive group. In order to elucidate whether the lack of SOX2 expression is an independent

Figure 3 Extensive lymphatic infiltration in a case of SOX2-negative esophageal squamous cell carcinoma (patient 1). (a) Overview of the tissue with cancer cells adjacent to primary invasive focus (arrows) and remote stroma observed mainly inside lymphatics (H&E). (b) Tumor cell nests are observed in dilated lymphatics in the lamina propria (H&E, $\times 200$). (c) Lymphatic invasion is observed in the muscularis propria (H&E, $\times 200$). (d) Immunostaining for D2-40 highlights vascular structures lined by endothelial cells around tumor cell nests ($\times 100$).

Table 4 Relationship between the expression of SOX2 and CD133 or p63 in squamous cell carcinomas of the esophagus*

	CD133			
	Negative	Focal	Moderate	Extensive
SOX2				
Negative	7	1	0	0
Focal	2	1	0	0
Moderate	6	5	2	1
Extensive	70	10	4	4
	p63			
	0	1+	2+	3+
SOX2				
Negative	0	1	0	7
Focal	0	0	1	2
Moderate	0	1	0	13
Extensive	0	4	2	82

Negative, no expression; focal, positive in 1–25% of cancer cells, moderate, 26–50%; extensive, > 50%. *No correlation found in either comparison ($P > 0.05$).

prognostic factor, a multivariate analysis was performed with the application of two other potential prognostic factors (pT classification and lymph node metastasis). Cox's proportional hazards model

identified the loss of SOX2 expression and nodal metastasis as independent poor prognostic factors for disease-specific survival (Table 5).

Gene Sequencing and Fluorescent *In Situ* Hybridization of SOX2

The entire exon, promoter, and 5'/3'-untranslated regions were successfully sequenced in all SOX2-negative cases using multiple sets of primers. All SOX2-negative cancers appeared to have the wild-type genotype. Fluorescent *in situ* hybridization was also conducted for the assessment of copy-number variations in SOX2-negative cancers. The numbers of SOX2 and centromere signals were similar in all cases, suggesting no copy-number loss of SOX2.

Promoter Methylation Assay

As neither mutations nor copy-number losses were found in SOX2, the loss of SOX2 expression was likely due to epigenetic gene silencing. Then, the methylation status of the SOX2 promoter domain was compared between SOX2-negative ($n=8$) and -positive cases ($n=4$). SOX2-positive cases were selected from samples with SOX2 immuno-expression. As shown in Figure 6, methylated alleles were

Figure 4 Duodenal biopsy from mucosal elevation detected during the follow-up (patient 2). (a) Tumor cell nests are observed in the lamina propria (arrows) (H&E, $\times 100$). (b) Immunostaining for D2-40 confirms that cancer cells are located inside lymphatics (arrows) ($\times 100$).

Figure 5 Post-operative survival curves of SOX2-positive and -negative cases. Patients with SOX2-negative esophageal cancer show significantly shorter recurrence-free (a) and disease-specific survival (b) than those with SOX2-positive malignancy ($P < 0.001$ and $P = 0.002$, respectively).

Table 5 A multivariate analysis for disease-specific survival

	OR (95% CI)	P-value
<i>SOX2 expression</i>		
Negative vs positive	7.05 (1.27–39.0)	0.025
<i>pT classification</i>		
pT1 vs pT2–4	1.28 (0.12–12.7)	0.830
<i>Lymph node metastasis</i>		
Present vs absent	8.35 (1.53–45.3)	0.014

observed in all SOX2-negative cases, but were scarce in SOX2-positive cancers. A semi-quantitative imaging analysis also revealed that the expression ratio of methylated to unmethylated alleles in SOX2-negative cancers was significantly higher than that in SOX2-positive cases, suggesting that *SOX2* promoter hypermethylation is a distinct molecular feature for SOX2-negative cancers.

Discussion

The results obtained in the present study are summarized as follows: (i) unlike the non-neoplastic squamous epithelium and most cases of esophageal cancers, a subset of esophageal squamous cell carcinomas (7%) completely lacked the expression of SOX2; (ii) SOX2-negative cancers were characterized by advanced stages and extensive lymphatic infiltration; (iii) multiple neoplastic lesions observed in SOX2-negative cases also lacked the expression of this transcriptional factor; (iv) the loss of SOX2 expression was an independent poor prognostic factor for patients with esophageal squamous cell carcinoma, and (v) *SOX2* promoter hypermethylation appeared to be a cause of *SOX2* silencing.

SOX2-negative squamous cell carcinomas of the esophagus had several unique histopathologic features. Lymphatic infiltration was observed in all patients, and was particularly extensive in 4/8 cases. One of these cases had recurrent cancer in the duodenum with mucosal elevation consisting of

Figure 6 Methylation-specific PCR analysis of the *SOX2* promoter. (a) PCR products recognizing unmethylated (U) and methylated (M) CpG sites were analyzed in *SOX2*-positive ($n = 4$) and -negative ($n = 8$) squamous cell carcinomas of the esophagus. Methylated products are observed in all *SOX2*-negative cases, but are scarce in *SOX2*-positive cancers. (b) The semi-quantitative imaging analysis reveals that methylated/unmethylated (M/U) ratios in *SOX2*-negative cases are significantly higher than those in *SOX2*-negative cancers ($P = 0.008$).

intra-lymphatic cancer cells. Similar to the present results, a previous study suggested that the stronger expression of *SOX2* negatively correlated with lymph node metastasis in squamous cell carcinomas of the esophagus.¹³ In addition, all multiple neoplastic lesions that developed in *SOX2*-negative cases lacked *SOX2* expression. This result may suggest that patients with *SOX2*-negative esophageal cancer have a susceptibility to this minor form of esophageal cancer. Furthermore, *SOX2* expression was already lost in intraepithelial neoplasia, indicating that the silencing of this gene participates in the early phase of carcinogenesis. Lastly, the expression of *SOX2* did not correlate with that of other biomarkers such as stem and cancer stem cell antigen CD133 or basal cell marker p63.¹⁴ Therefore, the lack of *SOX2* expression does not simply represent the loss of the stem/basal cell phenotype.

A potential relationship between *SOX2* expression and patient prognoses has been investigated in the most detail in squamous cell carcinomas of the lungs. The prognostic impact of the amplification of *SOX2* was not confirmed in two separate studies on pulmonary squamous cell carcinomas, while its overexpression on immunohistochemistry correlated with a better prognosis in other multiple cohorts.^{8,15–17} Findings obtained for esophageal squamous cell carcinomas are more limited and not conclusive. Although the index study suggested that the stronger expression of *SOX2* was associated with higher tumor grades and poorer prognoses,⁵ subsequent studies did not validate these findings.^{6,7} One potential cause for discrepant findings is that

different cut-off points were used for *SOX2* phenotyping. Our standard, in which only complete loss was defined as *SOX2*-negative, is the most stringent. *SOX2* expression was also lost in $\approx 20\%$ of esophageal adenocarcinomas, and negative expression was associated with a poor prognosis.¹⁸

According to the database cBioPortal (<http://www.cbioportal.org/index.do>), various malignant tumors have been found to harbor *SOX2* mutations, which randomly occur with no specific hot points. The highest incidence of *SOX2* mutations was observed in malignant melanoma (4%), followed by gastric cancer (3%), and cervical cancer (2%). *SOX2*-mutated adenocarcinomas of the esophagus have also been reported. As *SOX2* mutations are observed in various cancers, gene mutations were initially considered to be a potential cause of *SOX2* silencing in our study cohort. However, coding and non-coding regions were both the wild type in all *SOX2*-negative cases. *SOX2*-mutated squamous cell carcinomas of the esophagus were also not found in the database or a recent whole-exome sequencing study,¹⁹ suggesting that *SOX2* mutations are exceptional, even if they occur, in squamous cell carcinomas of the esophagus. The present study also indicated that copy-number loss was unlikely to be a cause of *SOX2* silencing.

Previous studies investigated *SOX2* promoter methylation in human malignant tumors. For instance, Wong and colleagues examined the methylation status of the *SOX2* promoter region in 72 endometrial carcinomas and found methylated alleles in 38% of cases.²⁰ Similar to the present study, *SOX2* methylation correlated with the shorter survival of patients with endometrial cancer.²⁰ Otsubo *et al* also reported that *SOX2* was hypermethylated in 12/74 (16%) gastric cancers, and methylation-positive cases showed significantly worse prognoses than methylation-negative cases.²¹ Taken together, the hypermethylation of *SOX2* in cancers appears to consistently correlate with an aggressive clinical course and poor prognosis. However, the effects of *SOX2* silencing may differ in mesenchymal cells. Alonso *et al* performed methylation-specific PCR on glioblastomas, and found that the *SOX2* promoter was hypomethylated in all patient samples relative to normal cell lines.²² *SOX2* was overexpressed in glioblastomas at both the protein and mRNA levels. In addition, ectopic *SOX2* expression induced the invasion and migration of glioma cells.²²

Since the expression of *SOX2* is considered to stimulate cellular proliferation, migration, invasion, and tumor metastasis in various human malignancies (eg, lung, esophageal, colorectal, prostate, and breast cancers),^{23–26} it remains unclear why the loss of *SOX2* expression is associated with highly malignant clinical phenotypes. One *in vitro* study reported that the exogenous expression of *SOX2* suppressed cell proliferation in normal and malignant gastric epithelial cell lines.²¹ A flow cytometry

analysis revealed that SOX2-overexpressing cells exhibited cell-cycle arrest and apoptosis, along with the weaker expression of cyclin D1 and phosphorylated Rb, and an increased p27 level.²¹ Therefore, SOX2-silenced cancer cells may escape cell-cycle arrest and become resistant to apoptosis.

In conclusion, SOX2-silenced squamous cell carcinomas of the esophagus appeared to be a minor, but distinct form of malignancy, characterized by extensive lymphatic invasion, a poor prognosis, and the development of multiple SOX2-negative neoplastic lesions. Hypermethylation of the promoter region was determined to be a critical epigenetic event leading to SOX2 silencing.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)