

SOX2 and Rb1 in esophageal small-cell carcinoma: their possible involvement in pathogenesis

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Clinicopathological features and pathogenesis of esophageal small-cell carcinoma remain unclear. We hypothesized common cellular origin and pathogenesis in small-cell carcinoma of esophagus and lung associated with SOX2 overexpression and loss of Rb1. Expression of squamous-basal markers (CK5/6 and p40), glandular markers (CK18 and CEA), SOX2, and Rb1 were evaluated in 15 esophageal small-cell carcinomas, 46 poorly differentiated squamous cell carcinomas, and 88 small-cell lung carcinoma, as well as 16 embryonic esophagus. Esophageal small-cell carcinoma expressed higher levels of glandular markers and lower levels of squamous-basal markers than poorly differentiated squamous cell carcinoma. No significant differences were observed in immunohistochemistry profiles between small-cell carcinoma of the esophagus and the lung. SOX2 expression was high in esophageal small-cell carcinoma (70% ± 33% of nuclei), small-cell lung carcinoma (70% ± 26%), and the embryonic esophagus (75% ± 4%), and it was significantly lower in poorly differentiated squamous cell carcinoma (29% ± 28%). Rb1 expression was significantly lower in esophageal small-cell carcinoma (0.3% ± 1%), small-cell lung carcinoma (2% ± 6%), and the embryonic esophagus (7% ± 5%), and it was significantly higher in poorly differentiated squamous cell carcinoma (51% ± 24%). The immunohistochemistry profiles of small-cell carcinoma of the esophagus and the lung are highly similar. The loss of Rb1 function is a key contributor to the pathogenesis of both neoplasms. In addition, SOX2 overexpression observed in esophageal and lung small-cell carcinoma as well as in the embryonic esophagus indicated that esophageal small-cell carcinoma may arise from embryonic-like stem cells in the esophageal epithelium. The two distinct differentiation patterns (neuroendocrine and glandular) of esophageal small-cell carcinoma further support the fact that SOX2 has a pivotal role in the differentiation of pluripotent stem cells into esophageal small-cell carcinoma cells.

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Esophageal small-cell carcinoma is a rare type of esophageal malignancy that accounts for only 0.05–3.1% of all esophageal carcinomas.^{1,2} Previous

studies reported that it is typically more aggressive than squamous cell carcinoma and is characterized by dissemination during the early stages of disease progression.^{3,4}

Neuroendocrine tumors occur in the esophagus at a low frequency compared with other sites in the gastrointestinal tract. The majority (>90%) of neuroendocrine tumors in the esophagus are high-grade neuroendocrine carcinomas, whereas low-grade tumors (G1, G2) are rarely observed in the

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esophagus.⁵ The association between neuroendocrine carcinoma and concomitant exocrine malignancies in the gastrointestinal tract has been well

Table 1 Clinicopathological features of 15 esophageal small-cell carcinomas and 46 poorly differentiated squamous cell carcinomas

	<i>Esophageal small-cell carcinoma, N = 15 (%)</i>	<i>Poorly differentiated squamous cell carcinoma, N = 46 (%)</i>	<i>P-value</i>
<i>Age</i>			
< 60	5 (33)	17 (37)	0.799
≥ 60	10 (67)	29 (63)	
<i>Gender</i>			
Male	11 (73)	41 (89)	0.134
Female	4 (27)	5 (11)	
<i>Location</i>			
Upper	2 (14)	7 (15)	0.261
Middle	5 (33)	25 (55)	
Lower	8 (53)	14 (30)	
<i>Size (mm)</i>			
< 50 mm	7 (47)	37 (80)	0.011
≥ 50 mm	8 (53)	9 (20)	
<i>pT</i>			
T1/2	5 (33)	24 (52)	0.205
T3/4	10 (67)	22 (48)	
<i>pN</i>			
N0	3 (20)	22 (48)	0.057
N1/2/3	12 (80)	24 (52)	
<i>Lymphovascular invasion</i>			
Absent	1 (7)	11 (24)	0.145
Present	14 (93)	35 (76)	
<i>TNM stage</i>			
0	0 (0)	0 (0)	0.097
IA	3 (20)	7 (15)	
IB	0 (0)	8 (17)	
IIA	0 (0)	6 (13)	
IIB	1 (7)	6 (13)	
IIIA	2 (13)	9 (20)	
IIIB	4 (27)	4 (9)	
IIIC	5 (33)	6 (13)	
IV	0 (0)	0 (0)	
<i>TNM Stage</i>			
I/II	4 (27)	27 (59)	0.031
III/IV	11 (73)	19 (41)	
<i>Preoperative treatment</i>			
None	13 (87)	18 (39)	0.001
Performed	2 (13)	28 (61)	
<i>Postoperative treatment</i>			
None	11 (73)	31 (67)	0.666
Performed	4 (27)	15 (33)	
<i>Treatment</i>			
Surgery only	10 (67)	12 (26)	0.005
Multimodality	5 (33)	34 (74)	

established, and a recent study demonstrated that the two malignancies share common biological and molecular features.^{6,7} However, there are relatively few reports describing esophageal neuroendocrine tumor. Previous studies evaluating admixtures of esophageal small-cell carcinoma and squamous cell carcinoma components have led some to hypothesize that esophageal small-cell carcinoma arises from stem cells that have undergone divergent differentiation.^{8,9} By contrast, another group proposed that esophageal small-cell carcinoma might originate from squamous cell carcinoma cells.¹⁰ However, the precise mechanisms mediating the development of esophageal small-cell carcinoma remain unclear. In lung, neuroendocrine cells and alveolar type 2 cells have been supposed to be progenitor and several molecules implicated in the pathogenesis of pulmonary neuroendocrine carcinoma.^{11–15} Among multiple genetic aberrations, loss of Rb1 protein expression is a critical regulator in neuroendocrine carcinoma, which might promote cell proliferation in neuroendocrine cells and the subsequent development of neuroendocrine tumors.^{11–14} SOX2 has also been reported to have a pivotal role in the development of small-cell lung carcinoma.¹⁶ SOX2 is a transcription factor required for the maintenance of pluripotency in embryonic stem cells¹⁷ and the self-renewal capacity of tissue-specific adult stem cells.^{18,19} SOX2 are upregulated in both small-cell and large-cell neuroendocrine carcinoma of the lung, whereas the SOX2 is undetectable in typical and atypical carcinoid tumors.^{19,20} In addition, SOX2 overexpression leads to an expansion of neuroendocrine cells in mice.¹⁵ Although not extensively studied, it is assumed that neuroendocrine cells are almost absent from the normal adult human esophagus. This poses the question of whether or not the pathogenesis of esophageal small-cell carcinoma is similar to its counterparts of the gastrointestinal tract, which harbors neuroendocrine cells in the mucosa. Esophageal small-cell carcinoma might originate from preexisting neuroendocrine cells or from different histological subtypes of malignant esophageal cells. The esophagus and respiratory tracts arise from the embryonic foregut. Therefore, we hypothesized that the loss of the Rb1 function and SOX2 protein expression might have an important role in the pathogenesis of esophageal small-cell carcinoma.

We attempted to identify differences in the clinicopathological features and immunohistochemistry profile differences between two types of esophageal malignancies (esophageal small-cell carcinoma/poorly differentiated squamous cell carcinoma) and then to investigate Rb1 and SOX2 protein localization in esophageal small-cell carcinoma.

Materials and methods

Tissue Samples

Surgically resected tissues from patients with esophageal small-cell carcinoma that had undergone surgery between January 1994 and December 2015 were obtained from six institutions in Japan (Tohoku University Hospital, Saitama Cancer Center, Nihonkai General Hospital, Iwate Prefectural Central Hospital, Iwate Prefectural Isawa Hospital, and Kesennuma City Hospital) (Table 1). The diagnosis of esophageal small-cell carcinoma was based on the histological criteria of the WHO 2010 classification system^{21,22} and the diagnosis was independently confirmed by three pathologists (AK, FF, and HS). The diagnosis was also confirmed by synaptophysin and/or chromogranin A immunostaining. The cases only positive for CD56 immunoreactivity, but not for other neuroendocrine markers, were excluded from this study. In cases for which multiple histological subtypes were observed, the diagnosis of esophageal small-cell carcinoma was made if >70% of the cells met esophageal small-cell carcinoma criteria.²¹ Exclusion criteria were as follows: (1) the tumor was located at the esophagogastric junction, or was associated with Barrett's esophagus or ectopic gastric mucosa, (2) tumor size decreased by more than two-thirds following neoadjuvant chemotherapy, or (3) no surgical specimen was available.

Tissues from 46 patients with poorly differentiated squamous cell carcinoma and 88 patients with small-cell lung carcinoma were also obtained. Poorly differentiated squamous cell carcinoma tissues were obtained from surgically resected cases, as small-cell carcinoma has been proposed to be a de-differentiated form of squamous cell carcinoma.¹⁹ The histopathological diagnosis of poorly differentiated squamous cell carcinoma was made independently by three pathologists (AK, FF, and HS). The diagnosis of poorly differentiated squamous cell carcinoma was made if the majority of the tumor tissue comprised round cells without apparent keratinization. The diagnosis of small-cell lung carcinoma was based on

the criteria described in the latest edition of the WHO classification system and positive immunostaining with at least one of the neuroendocrine markers evaluated (synaptophysin, chromogranin A, and CD56).²² The small-cell lung carcinoma specimens were reviewed independently by three pathologists (AK, YI, and HS). We also obtained 16 human embryonic (13 to 21 week) esophageal specimens (6 specimens from the cervical esophagus and 10 from unknown locations) and 16 noncancerous esophageal tissues. The noncancerous samples comprised tissues resected from patients with various benign conditions (schwannoma or aortoesophageal fistula) as well as esophageal stump tissue from patients who had undergone stomach resection.

The clinicopathological characteristics of the patients were carefully reviewed. The tumors were staged according to the 7th edition of the AJCC/UICC TNM-staging system for esophageal carcinoma²³ after the clinicopathological features of each case had been re-evaluated. Overall survival was defined as the time from the initial pathological diagnosis to the time of death or last censor. The study protocol was approved by the ethics committee of each participating institution.

Immunohistochemistry

The tissue samples were fixed with 10% formalin and paraffin-embedded as tissue blocks. Information about antibodies, antigen retrieval methods, and buffers used in the immunohistochemistry assays are summarized in Table 2. Immunohistochemistry procedure was based upon that described in a previous report.²⁴ Synaptophysin, chromogranin A, and CD56 were used as neuroendocrine markers, CK5/6 and p40 as squamous-basal markers^{25,26} and CK18 and CEA as glandular markers.^{27,28} A previously validated mouse monoclonal anti-SOX2 antibody (MAB4343, Clone 6F1.2, Merck Millipore, Darmstadt, Germany) was employed in this study.²⁹ Each sample was evaluated by two of the authors (HI and AK) using a previously established scoring

Table 2 Characteristics of the primary antibodies used in this study

Antibody	Clone	Vendor	Dilution	Antigen retrieval	Buffer pH
Synaptophysin	SY38	DAKO, Glostrup, Denmark	1/200	AC, 121 °C, 5 min	6.0
Chromogranin A	—	DAKO, Glostrup, Denmark	1/2000	None	—
CD56	1B6	Nichirei Biosciences Inc., Tokyo, Japan	1/1	AC, 121 °C, 5 min	6.0
CK5/6	D5/16B4	DAKO, Glostrup, Denmark	1/100	AC, 121 °C, 5 min	6.0
p40	—	EMD Millipore, Darmstadt, Germany	1/2500	AC, 121 °C, 5 min	6.0
CK18	DC-10	Novocastra Laboratories, Newcastle, UK	1/40	AC, 121 °C, 5 min	6.0
CEA	CEM010	Mochida, Tokyo, Japan	1/1500	None	—
TTF-1	8G7G3/1	DAKO, Glostrup, Denmark	1/100	AC, 121 °C, 5 min	6.0
Ki-67	MIB-1	DAKO, Glostrup, Denmark	1/100	AC, 121 °C, 5 min	6.0
SOX2	6F1.2	Merck Millipore, Darmstadt, Germany	1/200	MW, 95 °C, 15 min	6.0
Rb1	1F8	Gene Tex, California, USA	1/2000	AC, 121 °C, 5 min	8.0

Summary of antibodies used for immunohistochemistry in this study.

system ranging 0–12; calculated by multiplying the positivity (0: 0%, 1: <10%, 2: 10%–50%, 3: 51%–80%, or 4: >80% of cells positively stained) and the staining intensity (0: none, 1: mild, 2: moderate, or 3: strong staining).^{30,31} Positive immunoreactivity was defined as score ≥ 1 , and negative immunoreactivity defined as score 0.³¹ Nuclear staining of p40, Ki-67, TTF-1, SOX2, and Rb1 was quantitatively analyzed as the proportion of positively stained nuclei in an approximately 0.04 mm² (0.2 × 0.2 mm) region of each sample³² using the semi-automated image analysis software, Histoquest (TissueGnostics, Tarzana, Los Angeles, CA, USA).

Statistical Analyses

All statistical analyses were conducted using JMP Pro version 11.0.0 software (SAS Institute, Inc., Cary, NC, USA). Continuous variables were analyzed using Student's *t*-test or the Mann–Whitney *U*-test. Differences between clinicopathological factors and immunoreactivity were evaluated using Pearson's χ^2 -test, Fisher's exact test, or the Mann–Whitney *U*-test. Overall survival curves were constructed using the Kaplan–Meier method, and compared using the log-rank test. A *P*-value < 0.05 was considered statistically significant.

Results

Clinicopathological Features of Esophageal Small-Cell Carcinoma

Esophageal small-cell carcinoma represented 0.46% (4/867) of surgically resected esophageal carcinoma in a single institution (Tohoku University Hospital). The clinicopathological features of the 15 esophageal small-cell carcinoma patients and the 46 poorly differentiated squamous cell carcinoma patients were summarized in Table 1. The tumor size was larger ($P=0.011$) and TNM stage was higher ($P=0.031$) in esophageal small-cell carcinoma than in the poorly differentiated squamous cell carcinoma. Four patients in the esophageal small-cell carcinoma group received postoperative chemotherapy with cisplatin+etoposide, cisplatin+irinotecan, carboplatin+etoposide, or irinotecan, according to the regimen recommended for small-cell lung carcinoma. The overall 1- and 3-year survival rates in the esophageal small-cell carcinoma group were 57.8 and 8.3%, respectively, and the median survival duration was 15.0 months. Only one patient was living 5 years after surgical resection. Overall survival was significantly shorter in esophageal small-cell carcinoma than in poorly differentiated squamous cell carcinoma in the overall population ($P < 0.001$) and in the subgroup analyses of overall survival, according to disease stage ($P=0.017$ for Stage I/II patients and $P < 0.001$ for Stage III/IV patients) (Figure 1). Of the 15 esophageal small-cell carcinoma tissue samples, 4 (27%) presented with squamous cell carcinoma components (mixed type) and the

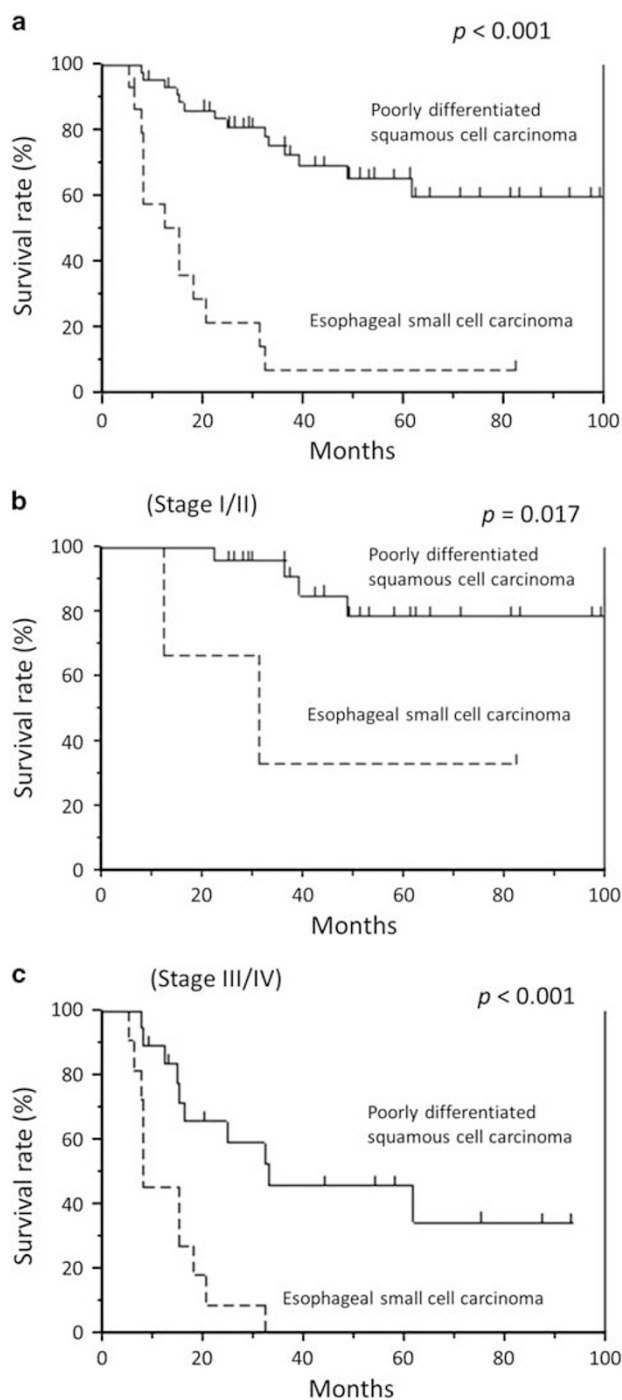


Figure 1 Overall survival of esophageal small-cell carcinoma patients compared with poorly differentiated squamous cell carcinoma. (a) Overall survival for esophageal small-cell carcinoma was significantly shorter than that for poorly differentiated squamous cell carcinoma ($P < 0.001$) among overall cases. In a Stage-stratified analysis, esophageal small-cell carcinoma demonstrated a worse prognosis than poorly differentiated squamous cell carcinoma ($P = 0.017$) in Stage I/II (b) and as well as in Stage III/IV ($P < 0.001$, (c).

remaining 11 samples (73%) were composed entirely of esophageal small-cell carcinoma components (pure type). There was no significant difference in overall survival between mixed type and pure type.

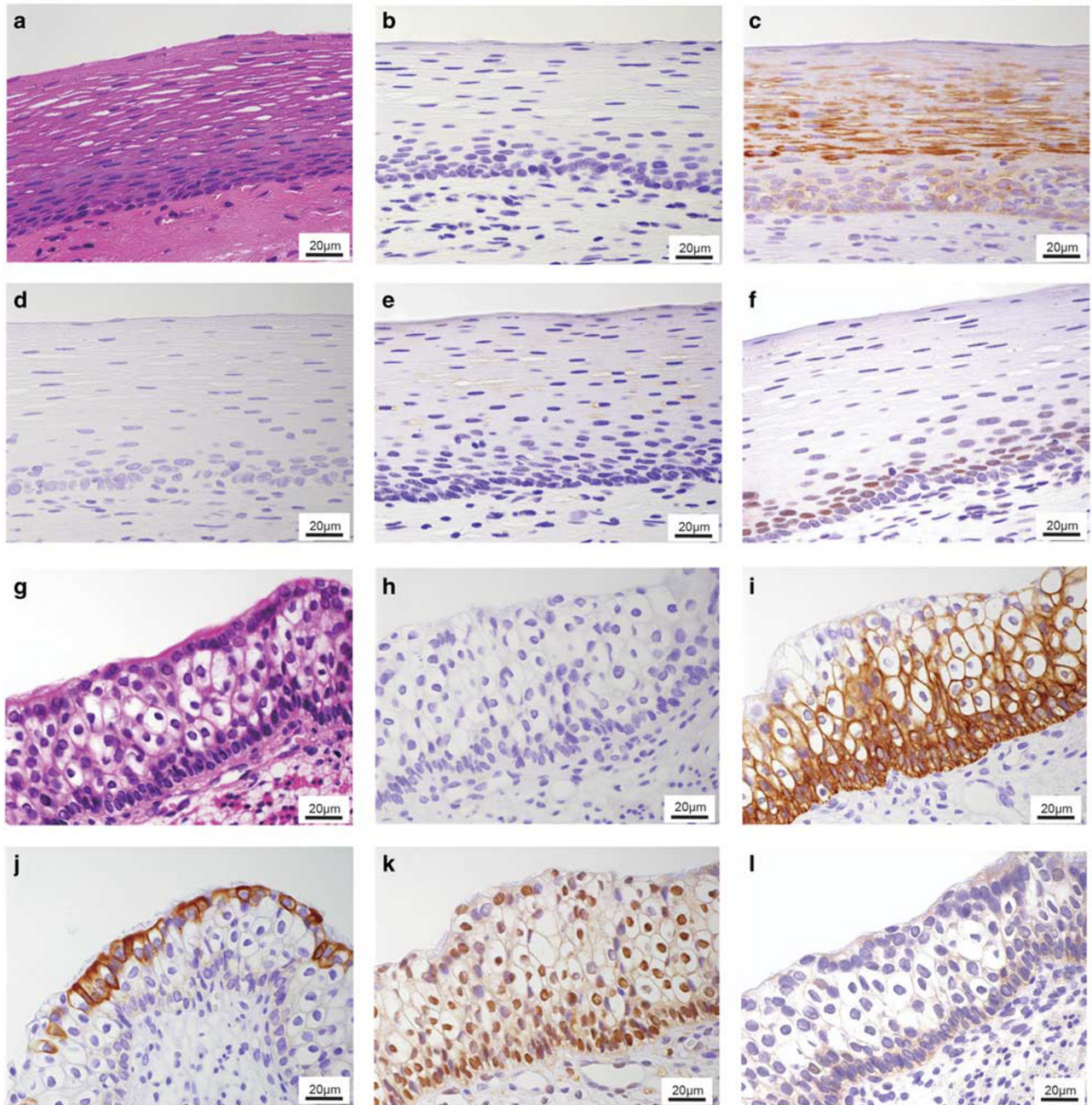


Figure 2 Representative hematoxylin and eosin-stained histopathological illustrations of the noncancerous esophagus covered by squamous epithelium (a) and the embryonic esophagus covered with stratified columnar ciliated epithelium (in week 20 embryo, g). No synaptophysin immunoreactivity was detected in both noncancerous and embryonic esophagus (b and h). CK5/6 immunoreactivity was diffusely detected in the noncancerous esophageal mucosa (c) but not in the basal to middle layer of the human embryonic esophagus (i). CK18 immunoreactivity was not detected in the noncancerous esophagus (d), whereas detected in the superficial layer of the embryonic esophagus (j). SOX2 immunoreactivity was not discernible in the noncancerous esophagus (e), whereas diffuse and marked immunoreactivity of SOX2 detected in the embryonic esophagus (k). Rb1 immunoreactivity was detected in the parabasal layer of noncancerous esophagus (f), whereas almost absent in the embryonic esophagus (l).

Noncancerous and Embryonic Esophageal Tissue Samples

Immunohistochemistry assays demonstrated that the noncancerous esophageal epithelium specimens expressed squamous-basal cells markers, but did not express synaptophysin, chromogranin A, or glandular markers (Figure 2a–d). The surface of

embryonic esophagus was covered by a stratified columnar ciliated epithelium until week 17, and a squamous epithelium became discernible in week 18 (Figure 2g). Expression of neuroendocrine markers was not detected in the embryonic esophageal epithelium (Figure 2h). Immunostaining for squamous-basal markers was observed in the basal

layer, and immunostaining for glandular markers was observed in the superficial layer of the embryonic esophagus (Figure 2i and j). SOX2 immunoreactivity was faint or absent in normal esophageal mucosa (Figure 2e), whereas a diffuse pattern of SOX2 expression was observed in the embryonic esophagus (Figure 2k). The proportion of SOX2-positive nuclei in embryonic esophageal tissues was $75\% \pm 4\%$ (mean \pm s.d.). Rb1 immunoreactivity was observed in the parabasal layer of noncancerous esophageal tissues ($52\% \pm 5\%$, Figure 2f) but was absent or faint in the embryonic esophagus ($7\% \pm 5\%$, Figure 2l).

Immunohistochemical Analysis in Esophageal Small-Cell Carcinoma, Poorly Differentiated Squamous Cell Carcinoma, and Small-Cell Lung Carcinoma

The results of the immunohistochemistry analysis of esophageal small-cell carcinoma and poorly differentiated squamous cell carcinoma tissues are summarized in Figure 3 and Table 3. Synaptophysin immunoreactivity was detected in all esophageal small-cell carcinoma samples (mean score of 6.7), whereas chromogranin A immunoreactivity was observed in 10 (67%) esophageal small-cell carcinoma samples (mean score of 2.4). Synaptophysin and chromogranin A immunoreactivity was not observed in any of the poorly differentiated squamous cell carcinoma samples. The expression of squamous-basal markers was significantly reduced in the esophageal small-cell carcinoma group, compared to the poorly differentiated squamous cell carcinoma group ($P < 0.001$ for both CK5/6 and p40). However, the expression of glandular markers was more frequently observed in the esophageal small-cell carcinoma group, compared with the poorly differentiated squamous cell carcinoma group. In the esophageal small-cell carcinoma group, CK18 staining was observed in all 15 (100%) samples ($P < 0.001$), and CEA staining was observed in 8 (53%) samples ($P = 0.003$). The Ki-67-labeling index was significantly greater in the esophageal small-cell carcinoma group ($75\% \pm 12\%$) than in the poorly differentiated squamous cell carcinoma group ($51\% \pm 20\%$) ($P < 0.001$). Similarly, the TTF-1-labeling index was $31\% \pm 36\%$ in the esophageal small-cell carcinoma group, compared with 0% in the poorly differentiated squamous cell carcinoma group. The SOX2-labeling index was also significantly greater in the esophageal small-cell carcinoma group ($70\% \pm 33\%$) than in the poorly differentiated squamous cell carcinoma group ($29\% \pm 28\%$) ($P < 0.001$), and the mean SOX2-labeling index in the esophageal small-cell carcinoma group was similar to the embryonic esophagus ($75\% \pm 4\%$) (Figure 4). The Rb1-labeling index was significantly lower in the esophageal small-cell carcinoma group ($0.3\% \pm 1\%$) than in the poorly differentiated squamous cell carcinoma group ($51\% \pm 24\%$) (Figure 5).

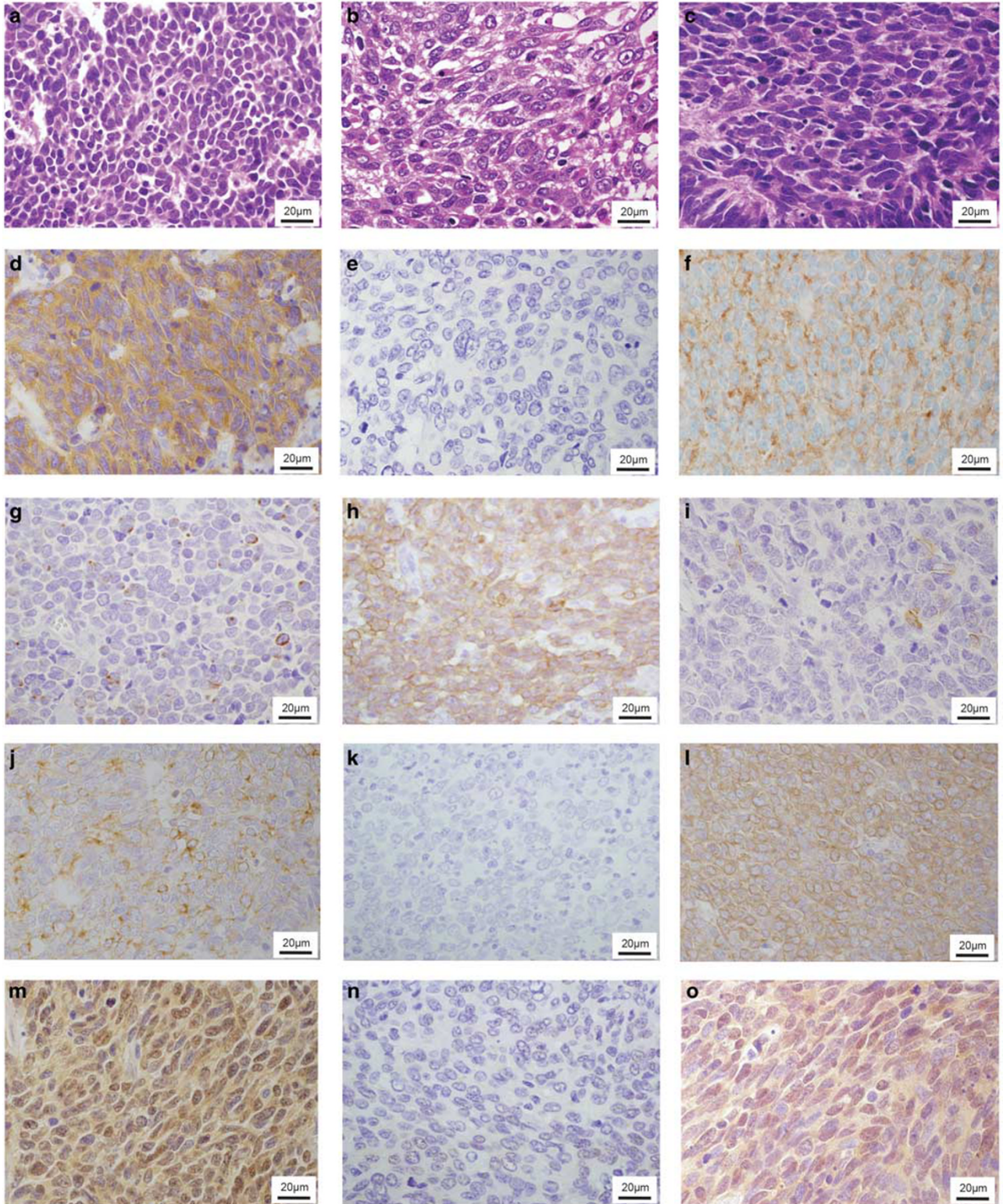
Immunohistochemistry analysis of the mixed type demonstrated that squamous-basal markers were expressed in squamous cell carcinoma components, whereas glandular markers and SOX2 were predominantly expressed in small-cell carcinoma components.

The immunohistochemistry profiles of the small-cell carcinoma of the esophagus and the lung were markedly similar. In both groups, squamous and basal cell markers were expressed at low or undetectable levels, and glandular markers were strongly expressed (Figure 3 and Table 3). The Ki-67-labeling index was similar in the esophagus and the lung groups (esophageal small-cell carcinoma, $75\% \pm 12\%$; small-cell lung carcinoma, $76\% \pm 17\%$, $P = 0.5$). The mean SOX2-labeling index in the small-cell carcinoma of the lung ($70\% \pm 26\%$) was similar to that of the esophagus ($70\% \pm 33\%$; $P = 0.377$) (Figure 4). Rb1 staining was nearly absent in both groups (esophagus, $0.3\% \pm 1\%$ and lung, $2\% \pm 6\%$; $P = 0.535$) (Figure 5).

Discussion

Takubo *et al.* reported that extrapulmonary small-cell carcinoma accounted for approximately 5% of small-cell carcinomas and that it most commonly presents in the gastrointestinal tract, especially in the esophagus.³³ Esophageal small-cell carcinoma is a highly aggressive disease with a poorer prognosis than that of squamous cell carcinoma.¹⁰ In this study, we excluded non-operative cases and only studied surgically resected patients. Therefore, this could be one of the reasons why the present cohort demonstrated rather favorable prognosis (median survival 15.0 months) compared with the results of previously reported studies of esophageal small-cell carcinoma (8 months by Yao *et al.* and 6 months by Pantvaidya *et al.*).^{3,4} However, it is still considered pivotal to demonstrate that the clinical outcome of esophageal small-cell carcinoma were significantly worse than that of poorly differentiated subtypes of squamous cell carcinoma even when curative surgery was performed. In addition, we revealed that the concomitant presence of squamous cell carcinoma components did not significantly influence the prognosis of esophageal small-cell carcinoma.³³

We also demonstrated that esophageal small-cell carcinoma and poorly differentiated squamous cell carcinoma are associated with distinct patterns of protein expression. We revealed that squamous-basal markers (CK5/6 and p40) are weakly expressed and glandular markers (CK18 and CEA) are more strongly expressed in esophageal small-cell carcinoma. Distinguishing esophageal small-cell carcinoma and poorly differentiated squamous cell carcinoma can be challenging, especially in small biopsy specimens, as the two malignancies frequently present with similar histological features, and neuroendocrine markers expressed in esophageal small-cell



carcinoma cells might not be prominent in cases of intra-tumoral heterogeneity. These differences might help establish a differential pathological diagnosis between the two neoplasms.

The pathogenesis of esophageal small-cell carcinoma remains controversial. One well-established hypothesis is that it arises from argyrophilic Kulchitsky cells, which are proposed to be present in both the bronchial and esophageal mucosa.^{34,35} However, despite the widespread acceptance of this hypothesis, the precise localization and distribution of neuroendocrine cells in the normal esophagus has not been thoroughly investigated. In the present study, neuroendocrine cells were absent in normal, noncancerous esophageal mucosa. Ho *et al.*³⁶ proposed that a pluripotent stem cell, rather than Kulchitsky cells, might be the common precursor of all epithelial neoplasms of the esophagus, including esophageal small-cell carcinoma. Some investigators proposed that the frequent coexistence of esophageal small-cell carcinoma and squamous cell carcinoma (16.7%) and adenocarcinoma (1.6%) indicates that esophageal small-cell carcinoma might originate from a pluripotent stem cell of endodermal origin that is capable of divergent differentiation.^{8,9,37} However, another group hypothesized that esophageal small-cell carcinoma might originate from squamous cell carcinoma cells in the esophagus.¹⁰ Using immunohistochemistry analysis, we observed two distinct patterns of cell differentiation (ie, neuroendocrine and glandular). These findings support the hypothesis of the divergent differentiation of pluripotent stem cells. Squamous cell carcinoma components are sometimes observed

in esophageal small-cell carcinoma specimens; however, the lack of squamous-basal marker expression in esophageal small-cell carcinoma is consistent with the hypothesis that esophageal small-cell carcinoma does not originate from squamous cell carcinoma cells, which comprise the most common histological subtype of esophageal cancers in Japanese patients. In addition, the morphology of tumor cells expressing glandular markers was distinct from both normal glandular cells and tumor cells that did not express glandular markers. Similarly, tumor cells expressing squamous-basal markers did not exhibit 'squamoid' histological features. Therefore, the discrepancies between the protein expression profiles and morphological features in esophageal small-cell carcinoma merit further investigation.

SOX2 is a well-known transcriptional regulator having crucial roles in maintenance of progenitor and neural stem cells and neuroendocrine differentiation.^{15,17,18} SOX2 was also reported to interact closely with other transcriptional factors (OCT4 and NANOG) and to function through phosphorylation, ubiquitination, SUMOylation, or acetylation of the molecule.³⁸ Multiple signaling pathways such as Notch, Shh, Wnt/ β -catenin pathways, and/or genes (eg, MASH1) were also reported to be involved in SOX2 regulation.^{15,39} Cell cycle regulators, such as p21, p16, or p53, were also reported as important molecular partners of SOX2; p21 (cyclin-dependent kinase inhibitor 1), a potent cell cycle regulator tightly controlled by p53, was reported to directly bind to SOX2 and negatively regulate its function.^{40,41} No direct association

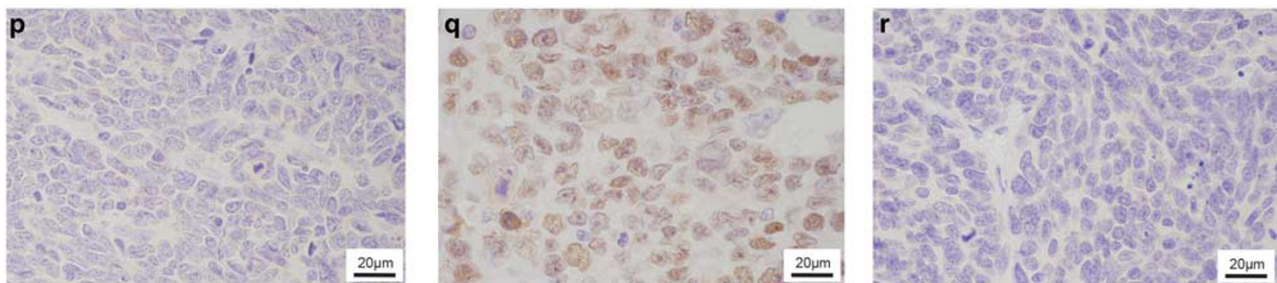


Figure 3 Continued

Figure 3 Histopathological and immunohistochemical features of esophageal small-cell carcinoma, poorly differentiated squamous cell carcinoma, and small-cell lung carcinoma. Hematoxylin–eosin staining of esophageal small-cell carcinoma (a), poorly differentiated squamous cell carcinoma (b), and small-cell lung carcinoma (c). All tumors are composed of small round-shaped cells with hyperchromatic nuclei and a high nuclear to cytoplasmic ratio (a). Synaptophysin immunoreactivity was detected in esophageal small-cell carcinoma (d) and small-cell lung carcinoma (e) but not in poorly differentiated squamous cell carcinoma (f). Focal and weak immunoreactivity of CK5/6 in esophageal small-cell carcinoma (g) and small-cell lung carcinoma (i), whereas diffuse and marked immunoreactivity of CK5/6 detected in poorly differentiated squamous cell carcinoma (h). Marked CK18 immunoreactivity in esophageal small-cell carcinoma (j) and small-cell lung carcinoma (l), whereas almost no immunoreactivity of CK18 detected in poorly differentiated squamous cell carcinoma (k). Diffuse and marked SOX2 immunoreactivity in esophageal small-cell carcinoma (m) and small-cell lung carcinoma (o), whereas SOX2 immunoreactivity in poorly differentiated squamous cell carcinoma was focal and weak (n). Rb1 immunoreactivity was virtually absent in esophageal small-cell carcinoma (p) and small-cell lung carcinoma (r) but detected in poorly differentiated squamous cell carcinoma (q).

Table 3 A summary of the immunohistochemical analysis of 15 esophageal small-cell carcinomas, 46 poorly differentiated squamous cell carcinomas, and 88 small-cell lung carcinomas

	Esophageal small-cell carcinoma, N = 15 (%)	Poorly differentiated squamous cell carcinoma, N = 46 (%)	Small-cell lung carcinoma, N = 88 (%)	P-value	
				Esophageal small-cell carcinoma vs Squamous cell carcinoma	Esophageal small-cell carcinoma vs Small-cell lung carcinoma
<i>Synaptophysin</i>					
pos	15 (100)	0 (0)	62 (71)	< 0.001	0.015
neg	0 (0)	46 (100)	26 (29)		
<i>Chromogranin A</i>					
pos	10 (67)	0 (0)	39 (44)	< 0.001	0.109
neg	5 (33)	46 (100)	49 (56)		
<i>CD56</i>					
pos	13 (87)	5 (11)	84 (95)	< 0.001	0.179
neg	2 (13)	41 (89)	4 (5)		
<i>CK5/6</i>					
pos	3 (20)	46 (100)	16 (18)	< 0.001	0.867
neg	12 (80)	0 (0)	72 (82)		
<i>p40</i>					
pos	1 (7)	46 (100)	3 (3)	< 0.001	0.546
neg	14 (93)	0 (0)	85 (97)	< 0.001	0.521
	(mean ± s.d.) 4 ± 16%	(mean ± s.d.) 81 ± 15%	(mean ± s.d.) 0.7 ± 4%		
<i>CK18</i>					
pos	15 (100)	18 (39)	85 (97)	< 0.001	0.468
neg	0 (0)	28 (61)	3 (3)		
<i>CEA</i>					
pos	8 (53)	7 (15)	46 (52)	0.003	0.939
neg	7 (47)	39 (85)	42 (48)		
<i>TTF-1</i>					
pos	7 (47)	0 (0)	62 (71)	< 0.001	0.07
neg	8 (53)	46 (100)	26 (29)	< 0.001	0.035
	(mean ± s.d.) 31 ± 36%	(mean ± s.d.) 0 ± 0%	(mean ± s.d.) 52 ± 44%		
<i>Ki-67</i>					
	(mean ± s.d.) 75 ± 12%	(mean ± s.d.) 51 ± 20%	(mean ± s.d.) 76 ± 17%	< 0.001	0.5
<i>SOX2</i>					
	(mean ± s.d.) 70 ± 33%	(mean ± s.d.) 29 ± 28%	(mean ± s.d.) 70 ± 26%	< 0.001	0.377
<i>Rb1</i>					
	(mean ± s.d.) 0.3 ± 1%	(mean ± s.d.) 51 ± 24%	(mean ± s.d.) 2 ± 6%	< 0.001	0.535

between SOX2 overexpression and loss of Rb1 protein has been reported, but results of previous studies above indicated that maintenance of progenitor cells through SOX2 was significantly associated with cell cycle and its regulatory molecules, including *Rb1* gene. SOX2 protein was also reported to be required in development of embryonic foregut endoderm, from which epithelial lining of multiple organs arises, eg, trachea, bronchi, alveoli, and esophagus. In addition, numbers of previous studies indicated that SOX2 had a key role in tumorigenesis

and controlled cancer stem-cell function in various human malignancies, although their functional and molecular mechanisms have not necessarily been fully elucidated.^{42–45}

In the human embryonic esophagus, squamous-basal markers (CK5/6 and p40) and glandular markers (CK18 and CEA) were expressed, but with different localization patterns. The squamous-basal markers were observed in the basal layer and the glandular markers in the superficial layer. SOX2 immunoreactivity was detected in all layers of the

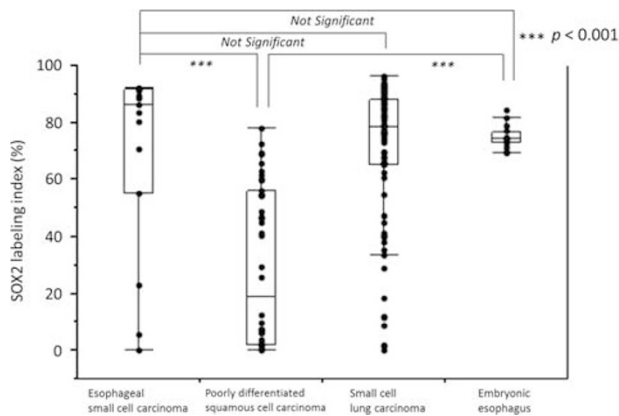


Figure 4 The percentage of SOX2-positive nuclei in esophageal small-cell carcinoma was almost equivalent to that in small-cell lung carcinoma and embryonic esophagus, and it was significantly higher than that in poorly differentiated squamous cell carcinoma ($P < 0.001$).

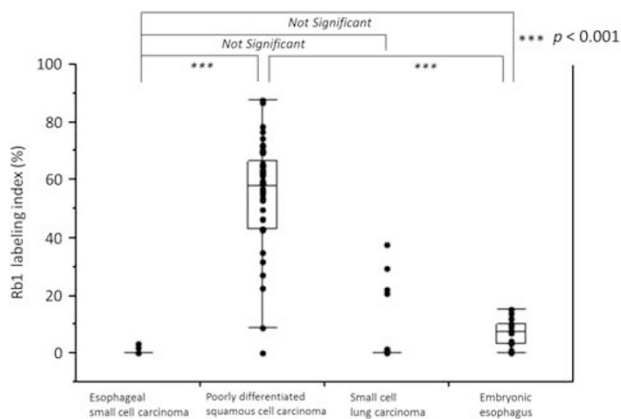


Figure 5 Rb1 was virtually absent in esophageal small-cell carcinoma, as in small-cell lung carcinoma and embryonic esophagus. The labeling index of Rb1 was significantly lower than that of poorly differentiated squamous cell carcinoma ($P < 0.001$).

embryonic esophagus, but not in noncancerous adult esophageal tissues. These findings indicate that SOX2 might contribute to the maintenance of pluripotency in human embryonic esophageal epithelial cells, and that SOX2 is degraded following the acquisition of a mature squamous esophageal mucosa cell phenotype.^{19,46} In the early embryonic lung and bronchial tissues, neuroendocrine bodies, which are associated with organogenesis, are in general considered as tumor progenitor of neuroendocrine neoplasms in the lung.⁴⁷ However, in our present study, neuroendocrine marker expression was not detected in the embryonic esophagus or in noncancerous esophageal mucosa. The absence of neuroendocrine marker expression in human embryonic esophageal tissues detected in this study appears to be inconsistent with SOX2 expression in the embryonic esophagus. Therefore, we propose the following two hypotheses for this finding; (1) The

regulators of SOX2 required for neuroendocrine differentiation in the synergistic fashion, such as MASH1, OCT4, NANOG, or Notch ligands and others may not be expressed in esophageal embryonic tissues and (2) SOX2 could contribute to neuroendocrine differentiation in much earlier embryonic stage of human esophageal development.^{17–19} However, further investigations such as the evaluation of more cases of human developing esophagus at different stages are required to further explore this interesting hypothesis.

The protein expression profile of esophageal small-cell carcinoma was similar to the embryonic esophagus, especially in columnar cells in the superficial layer. Specifically, both tissues strongly expressed SOX2 and glandular markers. SOX2 overexpression was reported to expand the population of neuroendocrine cells in mice and promote the maintenance of pluripotency in embryonic stem cells, indicating that SOX2 might induce neuroendocrine differentiation in embryonic-like pluripotent cells.¹⁵

Patterns of protein expression in small-cell carcinoma of the esophagus and the lung were markedly similar, specifically with respect to SOX2 overexpression and loss of Rb1 expression. In the lung, SOX2 expression and loss of Rb1 have been implicated in the pathogenesis via cell cycle regulation.^{11–14,16,19,48} Consistent with some previous reports, the results of the present study indicate that SOX2 might have a pivotal role in the development of small-cell lung carcinoma. Aberrations in *Rb1* represent an early molecular event in the pathogenesis, tumor development and the expression of neuroendocrine markers.

It is interesting that the great majority of the cases of esophageal high-grade neuroendocrine carcinoma are small-cell carcinoma and large cell neuroendocrine carcinoma relatively rare in esophagus. Recently, variable genetic alteration patterns have been reported in the pulmonary large cell neuroendocrine carcinoma, including ‘small-cell carcinoma-like subset’ characterized by *Rb1* and TP53 co-alteration.⁴⁹ Therefore, the loss of Rb1 expression could be a highly specific event in high-grade esophageal neuroendocrine carcinoma, in which the great majority of cases morphologically present as small-cell features but further investigations are required for clarification.

TTF-1 is also expressed in extrapulmonary small-cell carcinoma^{43,50–52} and its incidence in esophageal small-cell carcinoma in previous studies widely varies. In the present study, TTF-1-labeling index was significantly greater in the small-cell carcinoma of the lung than the esophagus, but this should be further clarified with a larger sample number.

The common embryonic origin (foregut) of the esophagus and lung, and the protein expression profiles of small-cell carcinoma arising in the two different organs support the hypothesis that a

common mechanism mediates the pathogenesis of the two neoplasms. Specifically, the high levels of SOX2 expression and the loss of Rb1 function are considered key mediators of the pathogenesis of esophageal small-cell carcinoma as well as small cell lung carcinoma.

In summary, esophageal small-cell carcinoma is a highly aggressive neoplasm associated with a poor prognosis, compared with poorly differentiated squamous cell carcinoma. The protein expression profile of esophageal small-cell carcinoma was distinct from poorly differentiated squamous cell carcinoma, as esophageal small-cell carcinoma strongly expressed glandular markers and SOX2, and weakly expressed squamous-basal markers and Rb1. However, strong SOX2 immunoreactivity and weak Rb1 immunoreactivity was observed in both esophageal small-cell carcinoma and small-cell lung carcinoma, as well as in the embryonic esophagus. Together, these results indicate that SOX2 overexpression and the loss of Rb1 protein expression might have a pivotal role in the divergent differentiation of pluripotent embryonic-like epithelial cells and the development of esophageal small-cell carcinoma.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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