Immunohistochemical analysis for cell proliferation-related protein expression in small cell carcinoma of the esophagus; a comparative study with small cell carcinoma of the lung and squamous cell carcinoma of the esophagus

K. Okudela[1], T. Ito[1], Y. Kameda[2], N. Nakamura[3] and H. Kitamura[1]

[1]Department of Pathology, Yokohama City University School of Medicine,

[2]Division of Pathology, Kanagawa Cancer Center Hospital and [3]Division of Pathology, Yokohama Citizen's Municipal Hospital, Japan

Summary. Small cell carcinoma is a rare neoplasm in the esophagus. To evaluate cell proliferation activity and its underlying mechanisms in this tumor, we examined immunohistochemically 5 cases of small cell carcinoma of the esophagus (SCCE) for expressions of tumor suppressor proteins, oncoproteins and cell proliferation markers including p53, $p21^{WAF1/CIP1}$, retinoblastoma (Rb) protein, bcl-2, Ki-67 and PCNA, and compared the results with those of 5 cases of small cell carcinoma of the lung (SCCL) and 10 cases of squamous cell carcinoma of the esophagus (SQCE). The prevalence and labeling index of p53-immunoreactivity tended to be higher in SCCE (4/5; 56.6%) and SCCL (4/5; 79.9%) than in SQCE (6/10; 48.8%). Expression of $p21^{WAF1/CIP1}$ was observed in 2 of 10 cases of SQCE. In contrast, its expression could not be detected in any cases of SCCE and SCCL examined. Expression of Rb protein was observed in 9 out of 10 cases of SQCE, but not in any cases of SCCE and SCCL. SCCE and SCCL showed more frequent and intense immunoreactivity for bcl-2 than SQCE. In expression of cell proliferation markers (Ki-67 and PCNA), no remarkable difference was observed among SCCE, SCCL and SQCE. These results suggest that SCCE and SCCL could share some genetic alternations including mutation of p53, loss of Rb gene and overexpression of bcl-2, and these may be related to the similar biological potentials between the two. Futhermore, SCCE was different from SQCE in expression of Rb protein and bcl-2, and these two types of esophageal carcinoma could arise through different molecular mechanisms.

Key words: Small cell carcinoma, Esophagus, Cell proliferation, Apoptosis

Offprint requests to: Koji Okudela, Department of Pathology, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa-Ku, Yokohama, 236-0004, Kanagawa, Japan. Fax: 81-45-789-0588

Introduction

Small cell carcinoma of the esophagus (SCCE) was first described by McKeown in 1952 and since then more than 200 cases have been reported in the literature (Casas et al., 1997). Small cell carcinoma, histologically indistinguishable from that of the lung, arises in a wide variety of extrapulmonary sites, such as the larynx, salivary gland, digestive tract, pancreas, uterus, prostate, urinary bladder, thymus, skin, breast and so on, but its incidence is very low when compared with that of the lung (Galanis et al., 1997). Extrapulmonary small cell carcinoma occurs not so infrequently in the esophagus, and its incidence is reported to be 0.05% to 9.0% of all esophageal tumors (Briggs and Ibrahim, 1983; Mori et al., 1989; Sasajima et al., 1990; Huncharek and Muscat, 1995; Galanis et al., 1997). The highest prevalence of SCCE is observed in East Asia (Briggs and Ibrahim, 1983; Mori et al., 1989). SCCE, like that of lung origin, is regarded to have a poor prognosis because of its rapid growth rate and early systemic dissemination (Briggs and Ibrahim, 1983; Mori et al., 1989; Huncharek and Muscat, 1995; Casas et al., 1997). The median survival after diagnosis reportedly ranges from 3.3 to 20.7 months (Briggs and Ibrahim, 1983; Mori et al., 1989; Casas et al., 1995; Huncharek and Muscat, 1995). Moreover SCCE, as is well known in that of the lung, also shows neuroendocrine differentitation (Springall et al., 1986; Mori et al., 1989; Sasajima et al., 1990; Tennvall et al., 1990; Hoda and Haidju, 1992; Nishimaki et al., 1993). Although the histogenesis of SCCE is still controversial, Ho et al. (1984) suggested that a totipotent primitive cell could serve as the common precursor for squamous cell carcinoma, adenocarcinoma and SCCE, because coexistence of each histological type is often seen. In clinical outcome, microscopic appearance and its neuroendocrine differentiation, SCCE has similar characteristics to small cell carcinoma of the lung

(SCCL).

p53 is one of the most common tumor suppressor proteins and abrogation of its function is considered to be a key event in tumor development. Cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$, which is one of the downstream factors of p53, mediates cell cycle arrest at G1 check point (Ullrich et al., 1992; Cox and Lane, 1995). Rb protein is also a common tumor suppressor, implicated in the regulation of cell cycle progression. An oncoprotein, bcl-2, is known to have an inhibitory effect on apoptotic cell death and confers a survival advantage to cells (Miyashita et al., 1994). PCNA is known as an auxilliary factor of DNA polymerase δ, which is strongly expressed by cells in late G1 to S phase of the cell cycle (Cox, 1997). While Ki-67 is expressed by cells in the cell cycle except in G0 phase (Cattoretti et al., 1992). PCNA and Ki-67 are generally used as cell proliferation markers. Alternation of p53, loss of Rb gene, and high expression of PCNA and Ki-67 have been reported to correlate with poor prognosis in several human malignancies (Taylor and Cote, 1997).

To the best of our knowledge, there have been few studies evaluating molecular characteristics of SCCE. To understand the mechanisms of development of SCCE, it is important to examine potential similarities or differences between SCCE and SCCL in the expression of regulatory proteins for cell proliferation. In the present study, we immunohistochemically examined 5 cases of SCCE for expressions of p53, p21$^{WAF1/CIP1}$, Rb protein, bcl-2, PCNA, and Ki-67, and compared the results with those of SCCL and SQCE.

Materials and methods

Specimens

Five cases of SCCE, 5 cases of SCCL, and 10 cases of SQCE including 5 with poor differentiation and 5 with good differentiation were examined. The specimens were surgically resected at Yokohama City University Hospital, Yokohama Citizen's Municipal Hospital, and Kanagawa Cancer Center Hospital between 1984 and 1996. None of the patients had undergone any anti-cancer chemo- or radiation-therapy before surgery. Clinical and histological information on SCCE is listed in Table 1.

Tissue preparation

Tissue samples were fixed with 10% buffered formalin, and routinely embedded in paraffin. The tissue blocks were cut into 5-μm thick sections and mounted on glass slides coated with 3-aminopropyl triethoxysilan (Shinetsu chemical, Tokyo, Japan). These sections were placed in a thermochamber at 37 °C overnight and used for hematoxylin eosin (HE) stain and immunohisto-chemistry.

Immunohistochemistry

The tissue sections were dewaxed, rehydrated, and immersed in 3% hydrogen peroxide/methanol for 10 min to block endogenous peroxidase activity. After a brief washing with distilled water, the tissue sections were immersed in 1M sodium citrate buffer (pH 6.8) and were heated at 99 °C for 30 min in a microwave oven to retrieve masked antigenic activity. After a brief washing with PBS, the tissue sections were incubated first with 5% normal goat serum for 30 min to block non-specific binding and then with mouse monoclonal antibodies against p53 (Novocastra, Newcastle upon Tyne, UK), p21$^{WAF1/CIP1}$ (Santa Cruz, Santa Cruz, California, USA), Rb protein (Novocastra), PCNA (DAKO, Glostrup, Denmark), Ki-67 (DAKO), and bcl-2 (DAKO) for 90 min at room temperature. After washing three times with PBS, the tissue sections were incubated with biotinylated goat anti-mouse IgG (E-Y Laboratories, North Amphlett Blvd., San Mateo, USA) as the secondary antibody for 60 min at room temperature. Immunoreactivity was visualized by the avidin-biotin-peroxidase complex method using diaminobenzidine (DAB) as the substrate. Nuclear counterstain was performed lightly with hematoxylin.

To confirm the neuroendocrine differentiation of SCCE and SCCL cells, immunostaining for general neuroendocrine markers, chromogranin A (DAKO), neuron specific enolase (Nichirei, Tokyo, Japan), and synaptophysin (DAKO) were performed in the same manner as described above except without the microwave treatment.

The immunoreactivity for p53, p21$^{WAF1/CIP1}$, Rb protein, Ki-67, and PCNA was judged positive when nuclei of one or more cancer cells in a nest showed

Table 1. Clinical information, histological subtype and immunoreactivity for neuroendocrine markers in 5 cases of SCCE.

CASE	AGE	SEX	LOCALIZATION	TNM CLASSIFICATION	HISTOLOGICAL SUBTYPE	NEUROENDOCRINE MARKER			SURVIVAL AFTER SURGERY
1	74	M	Im	T1 N0 M0	Combined	NSE (+)	Chr (+)	Syn (+)	9 months
2	72	F	Im	T3 N2 M0	Combined	NSE (+)	Chr (+)	Syn (+)	2 months
3	57	M	Im	T3 N4 M0	Oat cell	NSE (+)	Chr (+)	Syn (-)	8 months
4	70	M	Ei	T2 N2 M0	Combined	NSE (-)	Chr (+)	Syn (-)	6 months
5	71	F	Im	T1 N1 M0	Combined	NSE (+)	Chr (+)	Syn (+)	3 months

SCCE: small cell carcinoma of the esophagus; M: male; F: female; NSE: neuron specific enolase; Chr: chromogranin A; Syn: synaptophysin; Im: middle thoratic esophagus; Ei: lower thoratic esophagus; "Combined" means small cell carcinoma with foci of squamous cell carcinoma.

brown-colored signals. One thousand cells were randomly selected and the percentage of positive cells was used as a labeling index. The immunostaining intensity for bcl-2 was divided into four grades based on the following criteria according to Ohbu et al. (1997); 3 points (diffuse and strong reactivity), 2 points (diffuse and weak reactivity), 1 point (peripheral or focal reactivity), 0 points (negative). The immunoreactivity seen in the basal layer of the non-neoplastic esophageal epithelium was used as the standard for the evaluation of staining intensity.

Results

Histologically, SSCE had the typical appearance of small cell carcinoma characterized by a diffuse proliferation of cells with small round or oval-shaped hyperchromatic nuclei and narrow cytoplasm (Fig. 1a). Fig. 1b shows the histological appearance of well differentiated SQCE. All of the SCCE and SCCL showed a positive reactivity for at least one of the general neuroendocinre markers examined (Table 1). The results of immunohistochemistry for p53, $p21^{WAF1/CIP1}$, Rb protein, bcl-2, PCNA, and Ki-67 are summarized in Table 2. Immunoreactivity for p53 was seen in 4 of 5 cases of SCCE (80%), 4 of 5 cases of SCCL (80%), and 6 of 10 cases of SQCE (60%). Positive staining was seen in the nuclei of cancer cells (Fig. 2). The mean labeling index of p53 in positive cases of SCCE, SCCL and SQCE was 56.6%, 79.9%, and 48.8%, respectively (Table 2). A higher labeling index was observed in SCCE and SCCL than in SQCE. Immunoreactivity for $p21^{WAF1/CIP1}$ was seen in 2 out of 10 cases of SQCE (20%) with a mean labeling index of
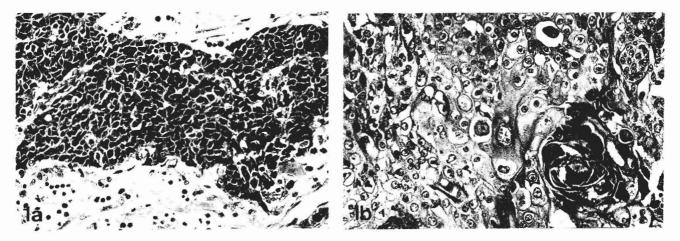

Fig. 1. Histological appearance of SCCE **(a)** and SQCE **(b)**. Both SCCE (a) have the typical histological appearance of small cell carcinoma characterized by diffuse proliferation of cells with small round or oval-shaped hyperchromatic nuclei, and narrow cytoplasm. (b); well differentiated SQCE. x 400
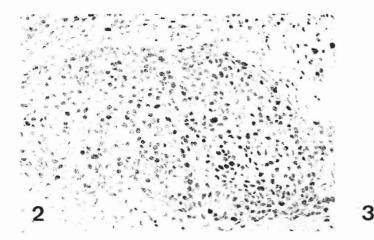

Fig. 2. Immunostaining for p53 in SCCE. Positive immunoreactivity is found in nuclei of cancer cells. Counterstained with hematoxylin. x 400

Fig. 3. Immunostaining for Rb protein in SQCE. A few nuclei of cancer cells show positive immunoreactivity. Counterstained with hematoxylin. x 400

5.1%, but not in any cases of SCCE and SCCL (Table 2). Two positive cases of SQCE were well differentiated carcinomas. Immunoreactivity for Rb protein was seen

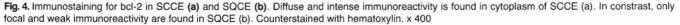**Table 2.** Results of immunohistochemistry.

ANTIGEN	SCCE	SCCL	SQCE
p53	56.6%, (4/5)	79.9%, (4/5)	48.8%, (6/10)
p21WAF1/CIP1	0%, (0/5)	0%, (0/5)	5.1%, (2/10)
Rb protein	*** (0/5)	***(0/5)	***(9/10)
PCNA	70.3%, (5/5)	77.8%, (5/5)	84.9%, (5/5)
Ki-67	52.3%, (5/5)	48.9%, (5/5)	53.5%, (5/5)
bcl-2	1.2 (5/5)	1.8 (5/5)	0.3 (3/10)

SCCE: small cell carcinoma of the esophagus; SCCL: small cell carcinoma of the lung; SQCE: squamous cell carcinoma of the esophagus; ***: not examined. Mean labeling index of immunoreactivity for p53, p21WAF1/CIP1, PCNA and Ki67 as a percentage (%), and mean intensity score for bcl-2 are described. () indicate the prevalance of positive immunoreactivity for each antigen.

in 9 of 10 cases of SQCE (90%) (Fig. 3), but was not present in any cases of SCCE and SCCL (Table 2). Immunoreactivity for bcl-2 was seen in all the cases of SCCE and SCCL, but was seen only in 3 of 10 cases of SQCE (30%). Diffuse and strong immunoreactivity was observed in 3 out of 5 cases of SCCE and in 3 out of 5 cases of SCCL (Fig. 4a). In contrast, 3 positive cases of SQCE showed only focal and week immuno-reactivity (Fig. 4b). The average staining score for bcl-2 was 1.2, 1.8 and 0.3 in SCCE, SCCL, and SQCE, respectively (Table 2). SCCE and SCCL tended to show higher prevalence and more intense immunoreactivity for bcl-2 than SCQE. The mean labeling index of Ki-67/PCNA in SCCE (Fig. 5a), SCCL, and SQCE (Fig. 5b) was 52.3%/70.3%, 48.9%/77.8%, and 53.5%/84.9%, respectively (Table 2). There were no significant differences in labeling index rates of these proliferation markers among SCCE, SCCL, and SQCE.
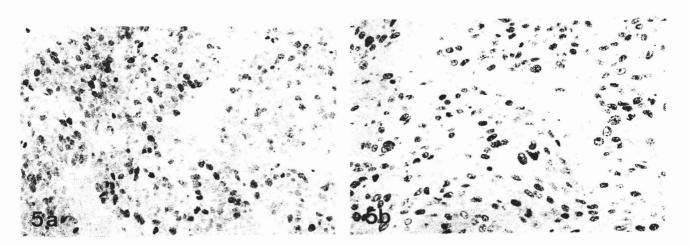

Fig. 4. Immunostaining for bcl-2 in SCCE **(a)** and SQCE **(b)**. Diffuse and intense immunoreactivity is found in cytoplasm of SCCE (a). In constrast, only focal and weak immunoreactivity are found in SQCE (b). Counterstained with hematoxylin. x 400

Fig. 5. Immunostaining for Ki-67 in SCCE **(a)** and SQCE **(b)**. Positive immunoreactivity is found in nuclei of both SCCE (a) and SQCE (b). Counterstained with hematoxylin. x 400

Discussion

The tumor suppresser protein p53 is known to mediate cell cycle arrest or apoptosis to avoid replicating mutant gene, resulting in suppression of tumor development (Ullrich et al., 1992; Cox and Lane, 1995) Abnormalities of p53 have been reported in more than 50% of human malignancies (Hollstein et al., 1991; Greenblatt et al., 1994). Wild type p53 is undetectable due to the small amount of protein under normal physiological conditions. Abnormal p53 resulting from gene mutation accumulates in the nuclei due to its longer half life than wild type protein. Thus, immunohisto-chemistry is a convenient method for detecting abnormality of p53. In the present study, 80% of SCCE, 80% of SCCL, and 60% of SQCE showed positive immunoreactivity for p53, with a labeling index of 56.6%, 79.9%, and 48.8%, respectively. Thus, higher prevalence and higher labeling index were seen in SCCE and SCCL than in SQCE. In esophageal carcinomas, the prevalence of immunoreactivity for p53 protein reported in the literature is very variable, ranging from 34% to 87% (Coggi et al., 1997). Korkolopoulou et al. (1993) reported that the prevalence of immunoreactivity for p53 was higher in small cell lung carcinomas (23/34; 67.7%) than non-small cell lung carcinomas (12/27; 44.4%). The results of the present study seemed to be consistent with the previous observations among lung carcinomas and suggested that there could be some differences in the prevalence of p53 abnormality among different histological types of esophageal carcinomas as among lung carcinoma.

A cyclin dependent kinase inhibitor $p21^{WAF1/CIP1}$, which is transcriptionally activated by p53, mediates cell cycle arrest at G1 check point to allow for repair of damaged DNA. Abnormal p53 is shown to be unable to transactivate $p21^{WAF1/CIP1}$. Disruption of the normal interaction between p53 and $p21^{WAF1/CIP1}$ has been observed in several human malignancies such as lung and pancreatic carcinomas (DiGiusppe et al., 1994; Marchetti et al., 1996; Hayashi et al., 1997). As in previous studies, the disruption of $p53$-$p21^{WAF1/CIP1}$ interaction was seen in the present study. Expression of $p21^{WAF1/CIP1}$ was not observed in any case of SCCE and SCCL examined, despite the high level of expression of p53. In contrast, weak expression of $p21^{WAF1/CIP1}$ was observed in a few cases of SQCE. These results suggest that the disruption of $p53$-$p21^{WAF1/CIP1}$ interaction could also be implicated in the carcinogenesis of esophageal carcinoma. $p21^{WAF1/CIP1}$ is implicated in not only cell cycle arrest, but in terminal differentiation of cells in several organs such as the hematopoietic system, colonic epithelium, and muscles, all in a p53-independent manner (Macleod et al., 1995; Parker at al., 1995; Schwaller et al., 1995; Zhang et al., 1995; Doglioni et al., 1996). Hayashi et al. (1997) reported that the higher level of expression of $p21^{WAF1/CIP1}$ was seen in better differentiated lung adenocarcinomas than in poorly differentiated ones. In the present study, the expression of $p21^{WAF1/CIP1}$ was observed only in well differentiated SQCE. This result suggests that $p21^{WAF1/CIP1}$ also plays a role in the tumoral differentiation of SQCE, as was seen in lung adenocarcinoma.

Loss of Rb gene has been demonstrated in a variety of human malignancies including lung and esophageal carcinomas, and is implicated in their carcinogenesis (Yokota et al., 1987; Xu et al., 1991; Jiang et al., 1993; Reissmann et al., 1993; Maesawa et al., 1994; Shibagaki et al.,1994; Kelley et al., 1995; Montesano et al., 1996). Furthermore, loss of Rb gene was reported to be correlated with high risk of metastasis in several human malignancies (Taylor and Cote, 1997). Immunohisto-chemistry is very convenient for monitoring the loss of both alleles of Rb gene (Jiang et al., 1993, Taylor and Cote, 1997). In the present study, the expression of Rb protein was not observed in any cases of SCCE and SCCL, but it was often seen in SQCE. Jiang et al. (1993) immunohistochemically demonstrated that loss of Rb gene was seen in 17% (6/36) of SQCE. It was reported that loss of Rb gene was seen in 90% of SCCL, but only in 15 to 30% of non-small cell carcinoma of the lung (Mori et al., 1990; Kelley et al., 1995). In the present study, a similar tendency was observed in esophageal cancers.

An onocoprotein, bcl-2, is thought to have an inhibitory effect on apoptotic cell death without affecting the progression of cell cycle (Miyashita et al., 1994). Alternation of bcl-2 gene was first detected in B cell lymphoma as t(14;18) chromosomal translocation. Since then, overexpression of bcl-2 has been immunohisto-chemically demonstrated in several human malignancies including esophageal and lung carcinomas (Ben-Ezra et al., 1994; Jiang et al., 1996; Ohbu et al., 1997). Ohbu et al. (1997) recently reported that 58% of SQCE (68/105) showed positive immunoreactivity for bcl-2, and when restricted to advanced cancers, positive immuno-reactivity was decreased to 42% (22/52). Jiang et al. (1996) reported that positive immunoreactivity for bcl-2 was observed more frequently and intensely in SCCL (104/111; 93.6%) than squamous cell carcinoma of the lung (23/64; 35.9%), and suggested a close correlation between bcl-2 expression and neuroendocrine differentiation in lung tumors. In the present study, more frequent and more intense immunoreactivity for bcl-2 was observed in SCCE and SCCL than in SQCE. These results confirmed previous observations and suggested the significance of overexpression of bcl-2 in the development not only of SCCL (Coppola et al., 1995), but of SCCE. Because overexpression of this onco-protein confers a survival advantage to neoplastic cells by blocking apoptotic cell death (Miyashita et al.,1994), common mechanisms may possibly be applied to cancers of a variety of organs. If this hypothesis is correct, higher expression levels of bcl-2 in SCCE and SCCL could be implicated in their rapid growth rate and early systemic dissemination.

PCNA and Ki-67 are generally used as cell proli-

feration markers to evaluate the growth rate of cancer cells. Strong expressions of these cell proliferation markers have been reported to correlate with poor prognosis in a variety of human malignancies (Korkolopoulou et al., 1993; Youssef et al., 1995). Korkolopoulou et al. (1993) reported in a previous study using 61 cases of lung carcinoma, that labeling index of PCNA tended to be higher in small cell carcinoma than in non-small cell carcinoma. Furthermore, it has been demonstrated that higher labeling index of these proliferating markers was correlated with a poor prognosis for SQCE (Morita et al., 1991; Morisaki et al., 1995; Youssef et al., 1995; Lam et al., 1996; Taylor and Cote, 1997). In the present study, however, no significant differences were observed in either labeling index of PCNA or Ki-67 among SCCE, SCCL, and SQCE.

In conclusion, SCCE showed similar characteristics to SCCL not only in histological appearance, but in genetic alternations. It may be considered that small cell carcinomas arising in different organs have many common genetic alternations, and these could contribute to their development and aggressive biological behavior.

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