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Frequent and Specific Involvement of Changes of the p16–RB Pathway in Esophageal Neuroendocrine Carcinoma

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Abstract. *Aim: This study investigated the immunohistochemical expression of retinoblastoma (RB) protein and p16 protein in 10 neuroendocrine carcinomas (NECs), in comparison to two mixed-type NECs; 28 squamous cell carcinomas (SCCs), and 12 carcinosarcomas (CSs) from patients with esophageal cancer. Materials and Methods: Immunohistochemical staining was performed using the avidin-biotin complex detection method. The staining was evaluated as diffusely positive, heterogeneous (in 5-95% of tumor cells), or diffusely negative. Results: The combination of a diffuse loss of RB and the diffuse overexpression of p16, which is found in highly aggressive malignant tumors and is considered to convincingly suggest changes in the p16–RB pathway, was found in all NECs (10/10). In contrast no mixed-type NECs, one SCC and one CS showed this finding. Coexisting intraepithelial carcinoma was detected in seven NECs and only one lesion showed the combination of diffuse RB loss and p16 overexpression. Conclusion: These data suggest that changes in the p16–RB pathway were universally and specifically involved in the development and invasion of esophageal NECs and that it may be a useful diagnostic marker and a potential therapeutic target.*

Neuroendocrine carcinomas (NECs) are poorly differentiated epithelial neoplasms with morphological and immunohistochemical features of neuroendocrine differentiation. All NECs are high-grade neoplasms of either small-cell NEC, which displays fusiform nuclei with finely granular chromatin, scant cytoplasm and nuclear molding, or large-cell NEC, which has round nuclei with prominent nucleoli and moderate amounts of cytoplasm. NECs can arise in most sites, including the gastrointestinal tract and pancreatobiliary system, and may be pure or mixed with variable amounts of adenocarcinoma, squamous cell carcinoma, or other components.

Esophageal NECs are exceedingly rare, accounting for 0.5-4.4% of esophageal malignancies (1-5) and highly aggressive malignant neoplasms. The median overall survival time of patients with esophageal NEC ranges from 8 to 15 months: most patients die within 2 years of diagnosis. Tumors with only regional extension can be treated by a combination of surgery, chemotherapy with platinum salts, and even radiotherapy (6), but in most cases, the disease is at an advanced stage at the time of diagnosis. Thus, further improvement of the diagnosis and treatment is required.

The comprehensive analysis of the molecular pathology of esophageal NEC and its clinical application have not been fully investigated due to the rarity of this entity: however, a recent study of 55 esophageal NECs showed a high rate of inactivating mutations in tumor protein p53 (*TP53*) and retinoblastoma (*RB*), as are seen in most cases of small-cell NEC, as well as in three other genes: notch homolog 1 (*NOTCH1*), protocadherin fat 1 (*FAT1*), and F-box/WD repeat-containing protein 7 (*FBXW7*) (7).

Some types of highly aggressive malignant tumors exhibit a combination of the diffuse loss of RB protein and the diffuse overexpression of p16 protein by immunohistochemical staining, which is regarded as a convincing finding suggesting changes in the p16–RB pathway (8). The purpose of this study was to immunohistochemically investigate the frequency of changes in the p16–RB pathway in esophageal NECs in comparison to mixed-type NECs, usual squamous cell carcinomas (SCCs) and carcinosarcomas (CSs) and investigate the status of such changes in their coexisting intraepithelial carcinomas.

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Key Words: Esophagus, Neuroendocrine carcinoma, p16–RB pathway, Immunohistochemistry, MiNEN.

Materials and Methods

The following esophageal specimens were analyzed: Seven surgically resected NECs, three biopsied NECs, two resected mixed-type NECs, 28 resected SCCs (randomly selected from among 759 SCC cases), and 12 resected CSs. All specimens were obtained from our hospitals from 2004 to 2018. For the diagnosis of NECs and mixed-type NECs, neuroendocrine differentiation was confirmed by immunohistochemistry using neuroendocrine markers chromogranin A, synaptophysin, and neural cell adhesion molecule (NCAM, CD56). All NECs were small-cell type. The two mixed-type NECs were a mixture of SCC and large-cell NEC and a mixture of adenocarcinoma and large-cell NEC. Probably preceding intraepithelial carcinoma (or dysplasia), which is solely a precursor preinvasive lesion and is not considered mixed-type NECs, were found to coexist in seven out of 10 cases of NEC. In addition to the three biopsied NECs, in six out of seven cases of resected NEC, an NEC component was detected in the initial biopsy, which indicated that the NEC component was not treatment-related in almost all cases. The patients with NEC did not develop NEC in association with a distinct clinical hormonal syndrome.

Immunohistochemical studies. Immunohistochemical staining was performed using the avidin-biotin complex detection method with a BenchMark automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). In each case, thin sections (3-μm) were cut from one or two formalin-fixed paraffin-embedded tissue blocks that included the maximum area of the tumor. These slices were stained with primary antibodies against RB (1:100 dilution; Leica, Newcastle upon Tyne, UK) and p16 (ready-to-use, E6H4TM; Ventana). The staining was evaluated as diffusely positive, heterogeneous (in 5-95% of tumor cells), or diffusely negative. Negative staining of the nuclei for RB indicated an abnormal loss of RB protein. Positive staining of the nuclei in the surrounding endothelial cells provided an internal control.

The combination of a diffuse loss of the nuclear expression of RB protein and the diffuse overexpression of p16 protein in the nucleus and cytoplasm (Figures 1 and 2) is found in highly aggressive malignant tumors with high proliferative activity [8] and the immunostaining pattern was considered to be a convincing finding that suggested changes in the p16–RB pathway in this study.

Statistical analyses. All the statistical analyses were conducted using JMP Pro Version 13.0.0 (SAS Institute Inc., Cary, NC, USA). Continuous variables were analyzed using Student's *t*-test, Pearson's chi-squared test or the Mann–Whitney *U*-test. Differences between clinicopathological factors and immunoreactivity were evaluated using Pearson's Chi-squared test, Fisher's exact test, or the Mann–Whitney *U*-test. Overall survival curves were constructed using the Kaplan–Meier method, and were compared using the log-rank test. Associations or differences with *p*-values of less than 0.05 were considered statistically significant.

Results

Comparison of the clinicopathological features. The characteristics of the patients and the lymph node status and stages of the resected cases according to the 8th Edition of the Union for International Cancer Control classification (9) are shown in Table I. The postoperative median survival time of the patients with NEC, mixed-type NEC, SCC and CS was 251, 347, 583, and 357 days, respectively, but did not differ significantly. There was no significant difference in the clinicopathological factors for NEC cases compared with all the other types (Table I).

Immunohistochemical features. Regarding RB immunostaining, all NECs showed diffuse and uniform nuclear negativity, *i.e.* diffuse loss of RB (Figures 1 and 2). Diffuse loss of RB expression was significantly more frequent ($p<0.05$) in NECs than in other types of tumors; the two mixed-type NECs were diffusely positive (normal) (Figure 3), and the majority of SCCs and CSs were diffusely positive or heterogeneous (Table II). Similarly, for p16 immunostaining, the nuclei and cytoplasm of all NECs exhibited diffuse and uniformly strong positivity, *i.e.* diffuse overexpression of p16 (Figures 1 and 2) and significantly ($p<0.05$) more frequently than other types of tumors; the two mixed-type NECs were diffusely negative (Figure 3), and the majority of SCCs and CSs were heterogeneous or diffusely negative (Table II).

The combination of the diffuse loss of RB and the diffuse overexpression of p16, suggesting changes in the p16–RB pathway was found in all 10 NECs. In contrast no mixed-type NECs (Figure 3), one SCC and one CS exhibited this profile (Table III).

The starting results for the seven coexisting intraepithelial carcinomas of NECs showed diffuse positivity for RB in six and diffuse negativity in one; diffuse positivity for p16 in two, heterogeneous positivity in one and negativity in four. The combination of the diffuse loss of RB and the diffuse overexpression of p16 in coexisting intraepithelial carcinoma was only found in one case (Table IV) (Figures 1, 4 and 5).

Discussion

The results of the current immunohistochemical study, which showed the nearly universal diffuse loss of RB protein in esophageal NECs, were in line with those of a recent molecular biological analysis that revealed a high rate of inactivating mutations in *RB* of 55 esophageal NECs (7). In addition, our study showed all such tumors were associated with the diffuse overexpression of p16 protein. RB and p16 are major components of the RB pathway, which controls the G1 checkpoint of the cell cycle. The disruption of the p16–RB pathway has been reported in various types of malignant tumors and the combination of the diffuse loss of RB and the diffuse overexpression of p16 is known to be found in highly aggressive malignant tumors with high proliferative activity, including NECs of various sites (8, 10-15). This combination was, even with the semi-quantitative assessment of immunohistochemical staining, regarded as a convincing finding that suggested changes in the p16–RB pathway (8).

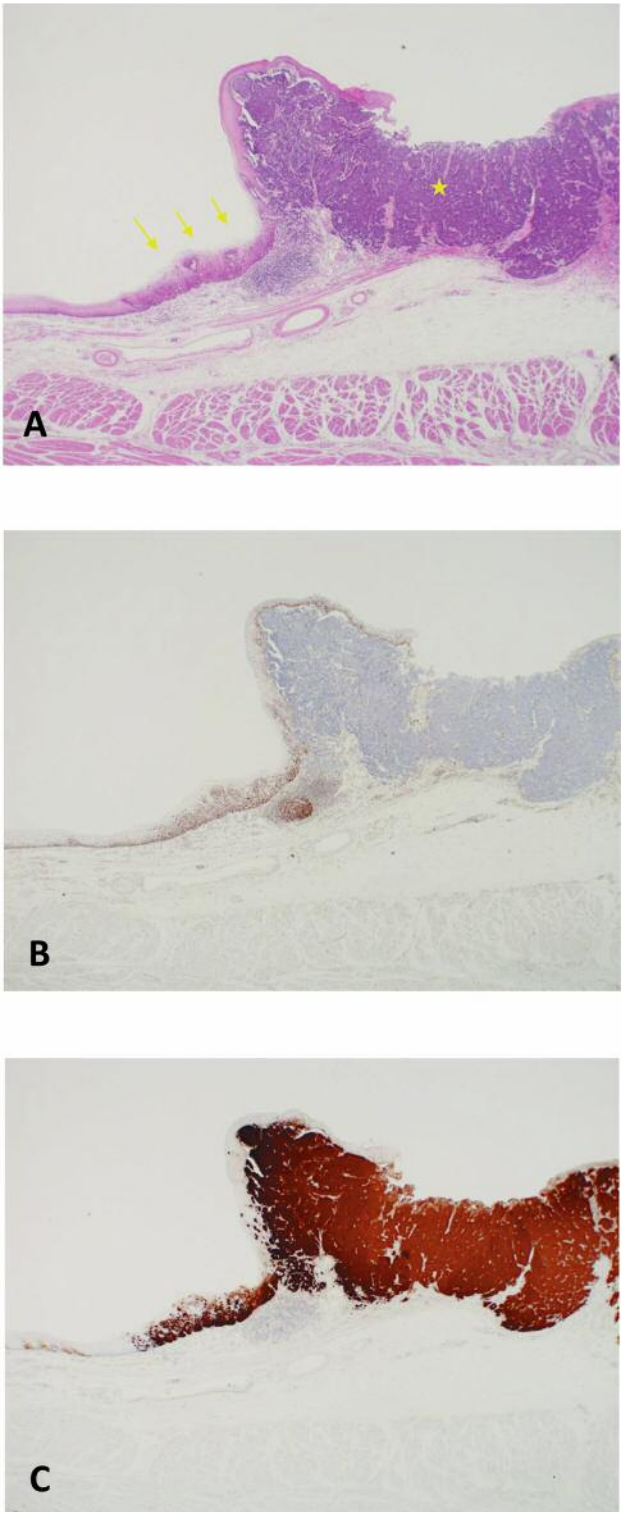

Figure 1. *Esophageal neuroendocrine carcinoma (NEC). A: Solid sheets of NEC cells (asterisk). Arrows indicate coexisting intraepithelial carcinoma. hematoxylin and eosin staining, ×12.5 B: Diffuse loss of retinoblastoma protein in NEC, but not in intra- epithelial carcinoma. RB immunostaining, ×12.5. C: Diffuse overexpression of p16 protein in NEC as well as in intraepithelial carcinoma. p16 immunostaining, ×12.5.*

In contrast to esophageal NECs, the combination of the diffuse loss of RB and the diffuse overexpression of p16 was significantly infrequent in other types of tumors, including mixed-type NECs, SCCs and CSs of the esophagus, suggesting that changes of the p16–RB pathway are more specifically and deeply involved in esophageal NEC.

In regard to p16, the overexpression of p16 protein in human tumors is primarily indicative of two situations: (i) The overexpression of p16 protein in benign or pre-malignant lesions with a low Ki-67 index, in which the overexpression is secondary to oncogene-induced senescence; and (ii) the overexpression of p16 protein in high-grade malignant lesions with a very high Ki-67 index, in which overexpression appears to be a mechanism that arrests uncontrolled proliferation caused by the failure of the RB pathway (secondary to viral infection, mutational silencing of the *RB* gene or other mechanisms) (8). p16 is the principal member of the cyclin-dependent kinase (CDK) inhibitor 2A family (INK4) and binds and inactivates CDK4/6, blocking the phosphorylation of RB and inducing cell-cycle arrest. The deregulation of RB results in the increased expression of p16 by tumor cells due to positive feedback (8).

Two mixed-type NECs in the present study were classified as mixed neuroendocrine non-neuroendocrine neoplasm (MiNEN), a conceptual category of mixed neoplasms, according to WHO 2017 (16). MiNENs are mixed epithelial neoplasms in which a neuroendocrine component is combined with a non-neuroendocrine component, each of which is morphologically and immunohistochemically recognizable as a discrete component and constitutes ≥30% of the neoplasm. Esophageal MiNENs usually consist of poorly differentiated NEC and either invasive SCC or adenocarcinoma (or both) (17), which is different from 'pure' NECs in which the non-neuroendocrine component consists solely of a precursor (preinvasive) neoplasm. One of our mixed-type NECs was composed of SCC and large-cell NEC, the other was composed of adenocarcinoma and large-cell NEC. Although the biological and pathological features of mixed-type NECs appear to overlap with those of NECs, the two mixed-type NECs in the present study did not show the combination of the diffuse loss of RB and the diffuse overexpression of p16, which may suggest that the mechanisms of tumorigenesis differ between esophageal NECs and mixed-type NECs. This is an interesting finding that has not been reported thus far; however, the number of cases was too small to draw definitive conclusions and further studies are required.

NECs at any site are usually associated with intraepithelial carcinoma (as a preceding component). In previous reports, intraepithelial carcinomas were detected in approximately 50% (1, 18, 19) of esophageal NECs. In our cases, coexisting intraepithelial carcinomas were found in seven of the 10 NECs. Interestingly, the diffuse positive expression of RB

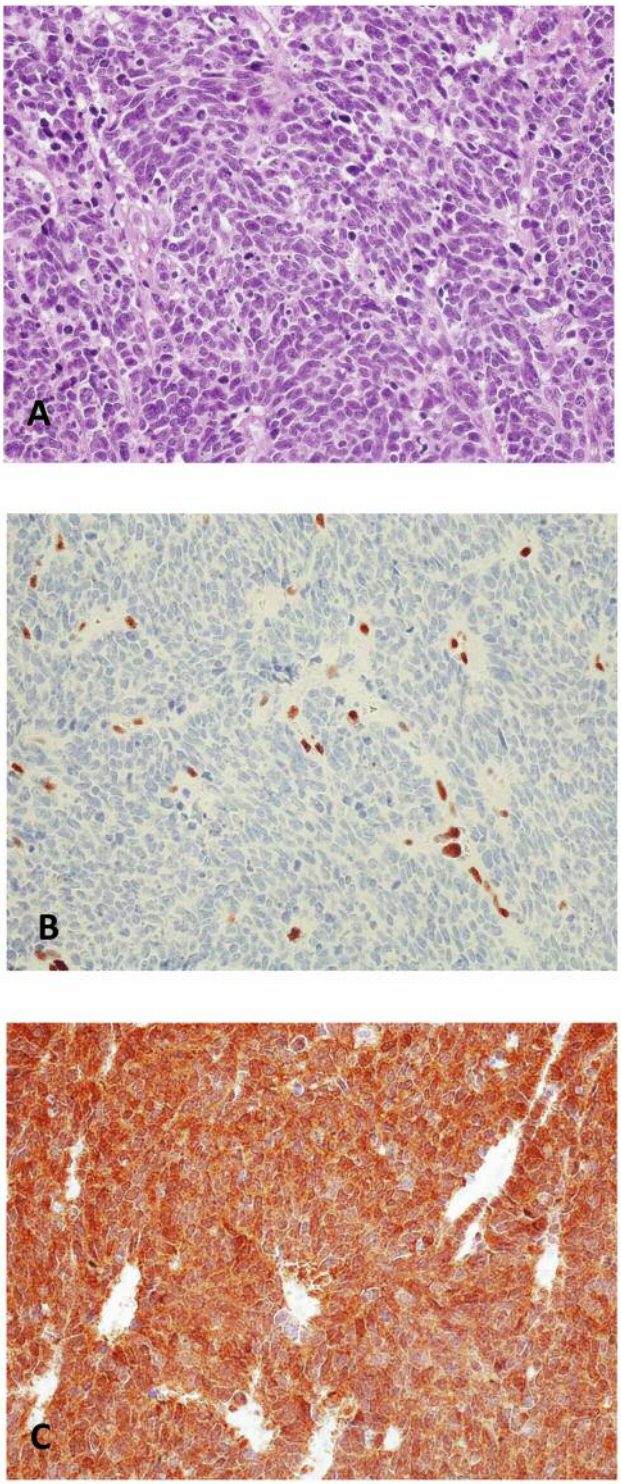

Figure 2. *Esophageal neuroendocrine carcinoma (NEC). A: Small-cell NEC of fusiform nuclei with finely granular chromatin, scant cytoplasm, and nuclear molding. Hematoxylin and eosin staining, ×400. B: Diffuse loss of nuclear expression of retinoblastoma protein. Note positive nuclear staining in endothelial cells as an internal control. Retinoblastoma immunostaining, ×400. C: Diffuse overexpression of p16 protein in the nucleus and cytoplasm. p16 immunostaining, ×400.*

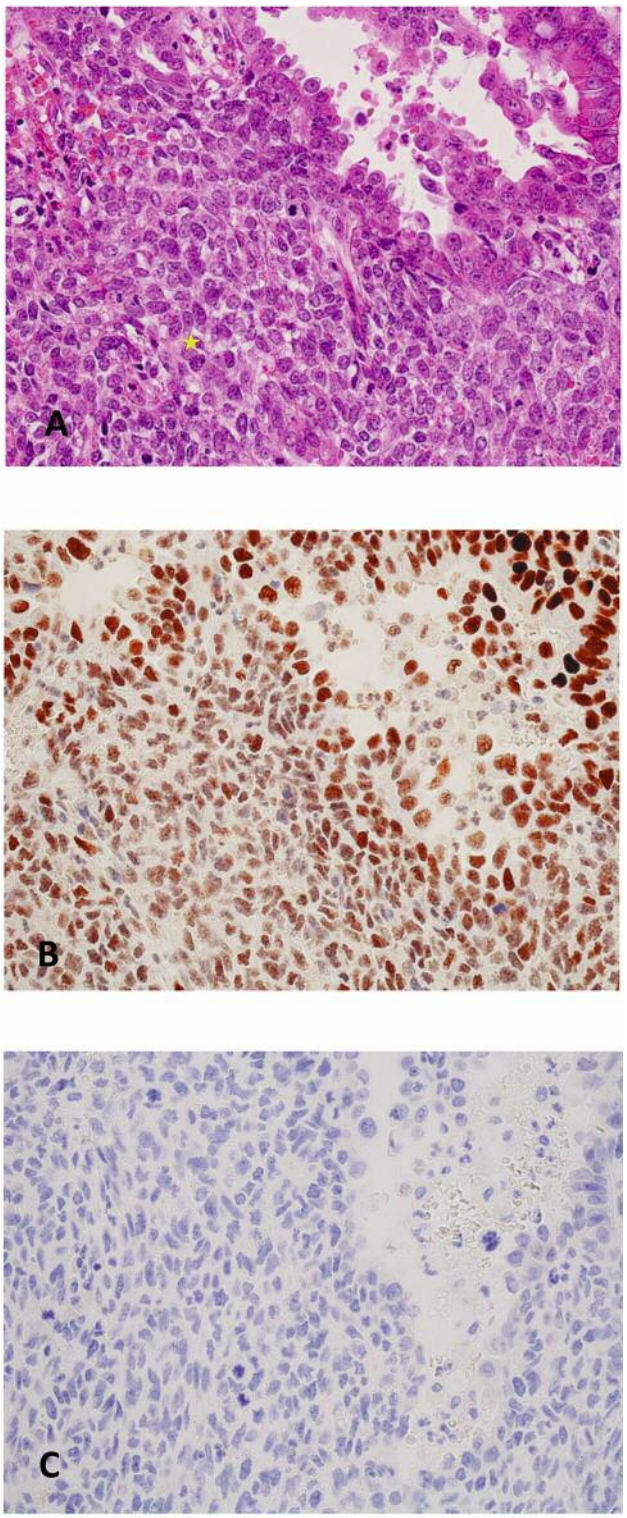

Figure 3. *Esophageal mixed-type neuroendocrine carcinoma (so-called MANEC). A: A mixture of tubular adenocarcinoma (upper right) and large-cell NEC (asterisk). hematoxylin and eosin staining, ×400. B: Diffuse positivity (no loss) of retinoblastoma protein. Retinoblastoma immunostaining, ×400 C: Diffuse negativity (no overexpression) of p16 protein. p16 immunostaining, ×400.*

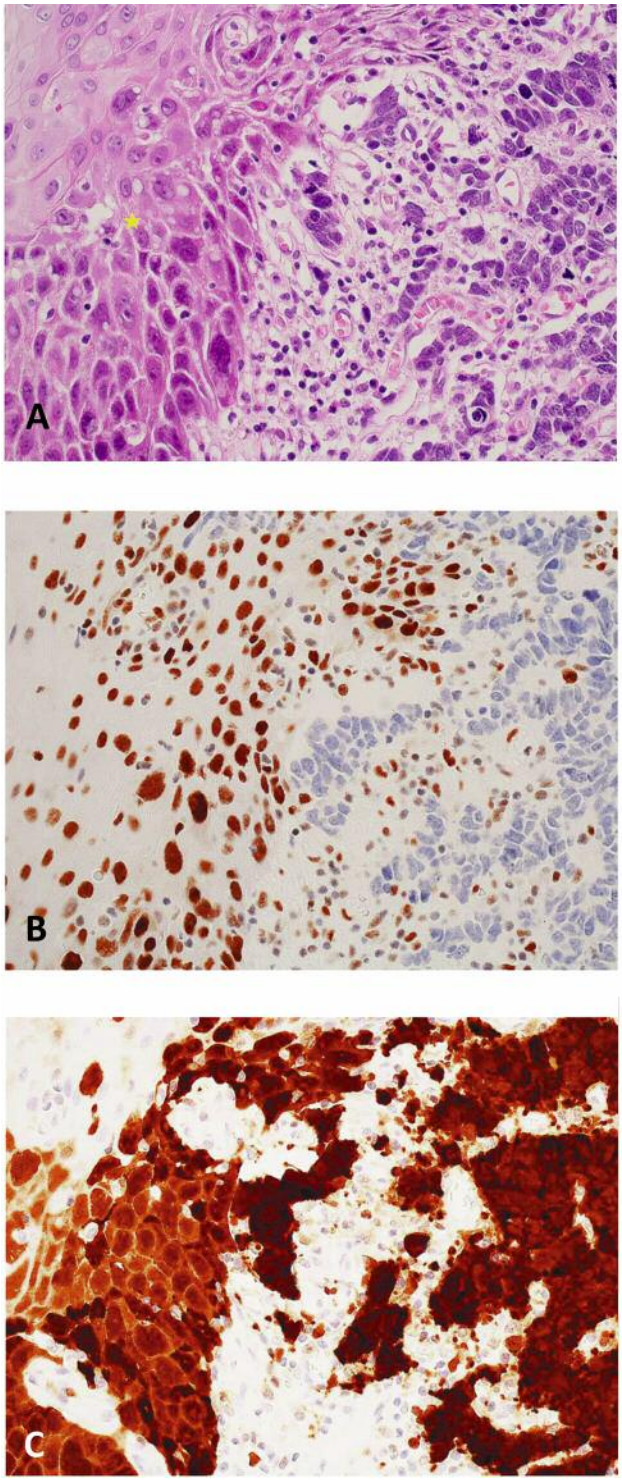

Figure 4. *Esophageal neuroendocrine carcinoma (NEC) and coexisting intraepithelial carcinoma. A: Small-cell NEC (right) and coexisting intraepithelial carcinoma (asterisk). Hematoxylin and eosin staining, ×400. B: Diffuse loss of retinoblastoma protein in NEC, but not in intraepithelial carcinoma. Retinoblastoma immunostaining, ×400. C: Diffuse overexpression of p16 protein in NEC as well as in intraepithelial carcinoma. p16 immunostaining, ×400.*

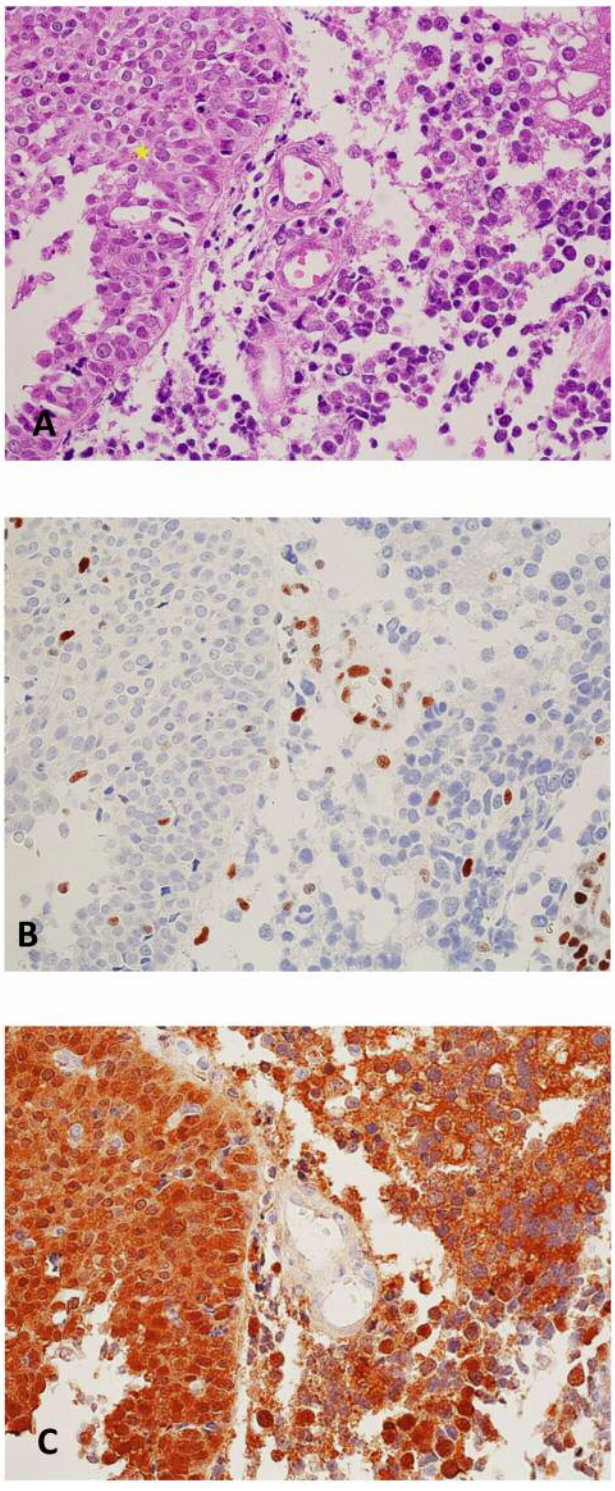

Figure 5. *Esophageal neuroendocrine carcinoma (NEC) and coexisting intraepithelial carcinoma. A: Small-cell NEC (right) and coexisting intraepithelial carcinoma (asterisk). Hematoxylin and eosin staining, ×400. B: Diffuse loss of retinoblastoma protein in NEC as well as in intraepithelial carcinoma. Retinoblastoma immunostaining, ×400. C: Diffuse overexpression of p16 protein in NEC as well as in intraepithelial carcinoma. p16 immunostaining, ×400.*

Table I. *Clinicopathological data of neuroendocrine carcinoma (NEC), mixed NEC, squamous cell carcinoma (SCC), and carcinosarcoma (CS).*

	NEC	Mixed-type NEC	SCC	CS	*p*-Value
N	10	2	28	12	NEC *vs*. other
Gender: Male:female, n	8:2	0:2	25:3	9:3	0.95
Mean age, years	69	66	69	68	0.46
Location: Upper/middle/lower, n	0/6/4	0/1/1	3/11/14	0/8/4	0.60
Mean tumor size, mm	48	25	42	68	0.79
Macroscopic type: Exophytic/ulcerated/unclassified, n	3/6/1	0/1/1	2/21/5	10/0/2	0.79
pT: 1/2/3/4, n	2/3/2/0*	1/0/0/1	0/5/22/1	1/8/2/1	0.45
pN: 0/1/2/3, n	3/2/1/1*	1/0/0/1	9/6/9/4	8/1/2/1	0.85
pStage: I/II/III/IV, n	2/1/2/0*	1/0/0/1	0/8/16/4	6/3/2/1	0.60
Postoperative median survival time, days	251*	347	583	357	0.15

*Except for three biopsy cases.

Table II. *Immunohistochemical study of p16 and retinoblastoma (RB) expression in neuroendocrine carcinoma (NEC), mixed NEC, squamous cell carcinoma (SCC), and carcinosarcoma (CS).*

		NEC	Mixed-type NEC	SCC	CS	*p*-Value
	Staining	n=10	n=2	n=28	n=12	
RB, n (%)	Diffusely positive	0 (0%)	2 (100%)	17 (61%)	4 (33%)	<0.05
	Heterogeneous	0 (0%)	0 (0%)	8 (29%)	5 (42%)	
	Diffusely negative (diffuse loss)	10 (100%)	0 (0%)	3 (11%)	3 (25%)	
p16, n (%)	Diffusely positive (overexpression)	10 (100%)	0 (0%)	1 (4%)	1 (8%)	<0.05
	Heterogeneous	0 (0%)	0 (0%)	8 (29%)	1 (8%)	
	Diffusely negative	0 (0%)	2 (100%)	19 (68%)	10 (83%)	

Table III. *Frequency of the combination of p16 and retinoblastoma (RB) immunohistochemical expression according to tumor type.*

			p16, n		
	Tumor type		Diffusely positive (overexpression)	Heterogeneous	Diffusely negative
RB, n	NEC (n=10)	Diffusely positive	0	0	0
		Heterogeneous	0	0	0
		Diffusely negative (diffuse loss)	10	0	0
	Mixed-type NEC (n=2)	Diffusely positive	0	0	2
		Heteroageneous	0	0	0
		Diffusely negative (diffuse loss)	0	0	0
	SCC (n=28)	Diffusely positive	0	6	11
		Heterogeneous	0	2	6
		Diffusely negative (diffuse loss)	1	0	2
	CS (n=12)	Diffusely positive	0	1	3
		Heterogeneous	0	0	5
		Diffusely negative (diffuse loss)	1	0	2

NEC: Neuroendocrine carcinoma; SCC: squamous cell carcinoma; CS: carcinosarcoma.

and the diffuse overexpression of p16 were observed in one and two out of seven of the intraepithelial carcinomas, respectively, rates that were considerably low in comparison to their infiltrated NEC areas (all cases). The combination of the diffuse loss of RB and the diffuse overexpression of p16 was only observed in one case of intraepithelial carcinoma. These findings suggest that the combination of the diffuse loss of RB and the diffuse overexpression of p16 make a

Table IV. *Frequency of the combination of p16 and retinoblastoma (RB) immunohistochemical expression in coexisting intraepithelial neoplasia (n=7) of esophageal neuroendocrine carcinoma.*

		p16, n		
		Diffusely positive (overexpression)	Heterogeneous	Diffusely negative
RB, n	Diffusely positive	1	1	4
	Heterogeneous	0	0	0
	Diffusely negative (diffuse loss)	1	0	0

critical contribution to the process of transitioning from intraepithelial carcinoma directly to invasive NEC in the esophagus.

In conclusion, our current immunohistochemical study demonstrated that the combination of a diffuse loss of nuclear expression of RB protein and the diffuse overexpression of p16 protein in the nuclei and cytoplasm was a nearly universal and specific finding in esophageal NECs. Changes in the p16-RB pathway may be a useful diagnostic and potential therapeutic target for esophageal NEC.

Conflicts of Interest

The Authors declare no conflicts of interest in association with this study.

Authors' Contributions

KF acquired, analyzed, and interpreted the patient data and was a major contributor to the drafting of the manuscript. NO conceived, designed and critically revised the article. KF, NO, TN, TI, and KK performed the histological and immunohistochemical evaluations of the patient specimens. KF, KO and MM collected the clinical data from the patient and critically revised the article. MT supervised the study. All Authors read and approved the final manuscript.

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