SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 40: 3772-3780, 2018

Copine 5 expression predicts prognosis following curative resection of esophageal squamous cell carcinoma

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Received April 8, 2018; Accepted September 13, 2018

DOI: 10.3892/or.2018.6742

Abstract. Patients with esophageal squamous cell carcinoma (ESCC) have a poor prognosis. Identification of biomarkers to accurately predict the risk of recurrence and survival following curative esophageal resection is required to improve patient outcomes. The copine 5 (*CPNE5*) gene encodes a calcium-dependent lipid-binding intracellular protein. Copine proteins interact with diverse target proteins that are components of pathways that aberrantly regulate the phenotypes of malignant cells. However, limited information is available on the role of *CPNE5* in cancer. The present study investigated whether *CPNE5* may serve as a predictive marker of the prognosis of patients with ESCC following curative resection. *CPNE5* mRNA expression levels and the methylation status of the *CPNE5* promotor region were measured in 11 ESCC cell lines. *CPNE5* mRNA expression levels in 106 pairs of surgically resected specimens were measured, and their associations with clinicopathological characteristics were analyzed. The *CPNE5* mRNA expression levels in 9 ESCC cell lines were decreased compared with those of the non-tumorigenic esophageal mucosa cell line Het-1A. Bisulfite sequencing detected the methylation of the *CPNE5* promotor region in all cell lines tested, including Het-1A. Furthermore, analysis of tissues revealed that *CPNE5* mRNA expression was significantly lower in ESCC cells compared with cognate non-cancerous adjacent mucosal cells. Kaplan-Meier analysis revealed that patients with low *CPNE5* expression experienced significantly shorter overall survival. Multivariable analysis identified low *CPNE5* expression to be an independent prognostic factor of OS. Analysis of recurrence patterns revealed that significantly more patients with local recurrence expressed lower levels of *CPNE5* mRNA. These findings indicated that *CPNE5* expression in ESCC tissues may serve as an informative biomarker for predicting ESCC recurrence, particularly in patients with local recurrence, and may help to ensure that patients receive optimal treatment and follow-up.

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Key words: esophageal cancer, copine 5, recurrence, biomarker

Introduction

Esophageal squamous cell carcinoma (ESCC) is associated with a considerable decline in quality of life, in addition to a poor prognosis (1,2). A primary goal of efforts to improve the management of the disease and patient outcomes is establishing methods to accurately predict the risk of recurrence, in addition to survival, following curative esophageal resection (3). Such information is urgently required to provide appropriate individualized perioperative follow-up and treatment (4,5). Furthermore, a better understanding of the molecular mechanisms of disease progression is essential, and identification of molecules that contribute to the pathogenesis of ESCC may lead to the development of novel biomarkers that facilitate precise risk stratification and monitoring of recurrence following esophagectomy (6,7).

The copine 5 (*CPNE5*) gene located on human chromosome 6p21.2 (8) belongs to the copine gene family, encoding calcium-dependent lipid-binding proteins comprising two N-terminal C2 domains (C2Ds) and a C-terminal A domain (9). CPNE5 localizes to the cytosol and is expressed at high levels in the brain, lymph nodes, testes and heart (10). *CPNE5* is expressed by differentiated neurons during neural development, suggesting that CPNE5 function is important for the function of the central nervous system (11). Furthermore, *CPNE5* expression is associated with alcohol dependence and obesity in Caucasians (12). *CPNE5* expression may be associated with the progression of ESCC, since copine proteins interact with diverse target proteins, including dual specificity mitogen-activated protein kinase kinase 1 (13), protein phosphatase 5 (14), and CDC42-regulated kinase (15), which are components of intracellular signaling pathways that influence the malignant phenotype (16). However, the role of *CPNE5* in cancer is unknown.

The present study assessed whether *CPNE5* may serve as a predictive marker of ESCC outcomes following curative resection. To answer this question, the expression of *CPNE5* mRNA

and the methylation of the *CPNE5* promoter was measured in ESCC cell lines and in surgically-resected ESCC tissues.

Materials and methods

Ethics approval and consent to participate. The present study rigidly adhered to the ethical guidelines of the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. Written informed consent for the use of clinical samples and data was obtained from all patients, as required by the Institutional Review Board of Nagoya University (Nagoya, Japan; approval no. 2014-0044).

Sample collection. ESCC cell lines (TE1, TT and TTn) and a non-tumorigenic epithelial cell line (Het-1A) were obtained from the American Type Culture Collection (Manassas, VA, USA). NUEC2 and WSSC cell lines were established at Nagoya University (17). KYSE510, KYSE590, KYSE890, KYSE1170, KYSE1260 and KYSE1440 cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan) (18). Cells were stored at -80˚C in a cell preservative (Cell Banker; LSI Medience Corporation, Tokyo, Japan) and cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an atmosphere containing 5% CO_2 at 37˚C. A total of 106 primary ESCC tissues and adjacent normal tissues were acquired from patients who underwent radical esophageal resection at Nagoya University Hospital between October 2001 and January 2016 (19). The tumors were determined to be radically resected when pathologically diagnosed as stage I to III. All tissue samples were histologically diagnosed as ESCC, immediately frozen following resection and stored at -80˚C. Specimens were histologically classified using the 7th edition of the UICC staging system for esophageal cancer (20). Patients who received neoadjuvant chemotherapy were excluded. Postoperative follow-up included physical examination, measurement of serum tumor markers every 3 months, and enhanced computed tomography of the chest and abdominal cavity every 6 months. Adjuvant chemotherapy was administered to selected patients according to their condition and at the discretion of the physician.

Analysis of CPNE5 mRNA expression levels. The expression levels of *CPNE5* mRNA were measured using a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA isolated using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) from cell lines and 106 pairs of surgically-resected primary ESCCs and adjacent normal tissues served as template for cDNA synthesis. Reverse transcription was performed as follows: 10.5 μl 1 μg/μl RNA, 4 μl of 5X first strand buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 μl 100 mM dithiothreitol (Thermo Fisher Scientific, Inc.), 1 μl 10 mM dNTP mix (Promega Corporation, Madison, WI, USA), 1 μl random primer (Roche Diagnostics, Basel, Switzerland), 1 μl 200 U/μl Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Inc.) and 0.5 μl RNase inhibitor (Roche Diagnostics) were mixed and incubated for 60 min at 37˚C. *GAPDH* mRNA expression levels (TaqMan; GAPDH control reagents; Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were quantified, and the data were used to normalize the expression levels. RT-qPCR was performed using the SYBR Green PCR Core Reagents kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) as follows: One cycle at 95˚C for 10 min; 40 cycles at 95˚C for 5 sec and 60˚C for 60 sec without a final extension step. The samples were tested in triplicate, and samples without a template were included in each PCR plate as a negative control (21). Real-time SYBR Green fluorescence was detected using an ABI StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) (22) and the $2^{-\Delta\Delta Cq}$ method was used for PCR quantification (23). The expression level of each sample is expressed as the value of the *CPNE5* amplicon divided by that of *GAPDH*. The sequences of the specific primers are listed in Table I.

Western blot analysis. The protein was extracted from each cell line using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.) and protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). For SDS-PAGE, 20 μg protein was added to a NuPAGE 4-12% Bis-Tris Gel (Thermo Fisher Scientific, Inc.) and electrophoresed for 35 min a 200 V. A polyvinylidene difluoride membrane was used for blotting and the membrane was blocked with 5% skim milk (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 60 min at room temperature. The CPNE5 protein expression levels in ESCC cell lines were evaluated with a rabbit anti-CPNE5 polyclonal antibody (1:100 dilution and overnight incubation at 4˚C; cat. no. HPA031369; Atlas Antibodies AB, Bromma, Sweden) as a primary antibody and anti-rabbit IgG HRP-linked antibody (1:1,000 dilution and 60 min incubation at room temperature; cat. no. 7074S; Cell Signaling Technology, Inc., Danvers, MA, USA) as a secondary antibody. As an internal control, β-actin protein expression was detected with a mouse anti-β-actin polyclonal antibody (1:10,000 dilution and incubated for 60 min at room temperature; cat. no. ab6276; Abcam, Cambridge, UK) as a primary antibody and anti-mouse IgG HRP-linked antibody (1:1,000 dilution and 60 min incubation at room temperature; cat. no. 7076S; Cell Signaling Technology, Inc.) as a secondary antibody. Enhanced Chemiluminescence Western Blot Analysis System (GE Healthcare, Chicago, IL, USA) was used for visualization of the secondary antibody. An ESCC cell line with relatively high *CPNE5* mRNA expression (KYSE590) and a low-expression ESCC cell line (TT) were evaluated.

Bisulfite nucleotide sequencing. Genomic DNA was isolated from the cell lines using a QIAamp DNA Mini kit (Qiagen GmbH) and treated with bisulfite (2 cycles at 95˚C for 5 min and 60˚C for 10 min). Bisulfite-modified DNA from ESCC cell lines and a non-cancerous esophageal mucosa cell line (Het-1A) were amplified as follows: One cycle at 94˚C for 2 min; and 50 cycles at 94˚C for 15 sec, 56˚C for 15 sec and 72˚C for 30 sec, using specific primers (Table I). Sequencing was performed by Eurofins Genomics Tokyo Co., Ltd. (Tokyo, Japan), using a Big Dye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Inc.) and a 3730x l DNA Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.) (24).

Table I. Primers and annealing temperatures.

Gene	Experiment	Type	Sequence (5'-3')	Product size (bp)	Annealing temperature
CPNE5	RT-qPCR	Forward	CATGTTTTCCAAGTCCGACC	106	60˚C
		Reverse	ATTGAGCGTGTTGTCGATGA		
	Bisulfite sequencing	Forward	GGTAGGAGTTTTTAGATTTGGAGGT	172	56˚C
		Reverse	ATTTCCCAATAACCCAAATAAAATC		
GAPDH	RT-qPCR	Forward	GAAGGTGAAGGTCGGAGTC	226	60˚C
		Probe	CAAGCTTCCCGTTCTCAGCC		
		Reverse	GAAGATGGTGATGGGATTTC		

CPNE5, copine 5; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; bp, base pairs.

Immunohistochemistry. Immunohistochemical staining was performed to determine the difference in CPNE5 protein expression between cancerous tissue and non-cancerous tissues in 45 representative clinical cases. Sections were incubated for 16 h at 4˚C with a rabbit polyclonal antibody raised against CPNE5 (cat. no. HPA031369; Atlas Antibodies AB) diluted 1:100 in Antibody Diluent (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Sections were incubated with secondary antibody (SignalStain® Boost IHC Detection Reagent labelled with HRP; Cell Signaling Technology, Inc.) for 30 min at room temperature. Antigen antibody complexes were visualized by exposure with liquid 3,3'-diaminobenzidine (Nichirei, Tokyo, Japan) for 2 min. A total of two independent observers evaluated the specimens using an optical microscope with x400 magnification as follows: Cancerous tissue >non-cancerous tissue; equivalent; or cancerous tissue <non-cancerous tissue.

Statistical analysis. Quantitative data are presented as the mean ± standard deviation. Patients were divided into low and high *CPNE5* groups according to the median levels of *CPNE5* mRNA expression in the cancerous tissues. The differences between *CPNE5* mRNA expression values in the two groups (differentiated cell lines vs. undifferentiated cell lines, or cancerous tissues vs. non-cancerous tissues) were compared using the Mann-Whitney test. The χ^2 test was used to analyze the association between *CPNE5* mRNA expression levels and clinicopathological characteristics. Overall survival (OS) and disease-free survival (DFS) rates were calculated using the Kaplan-Meier method. The Cox proportional hazards model was used to compare survival rates, and multivariable regression analysis was used to identify prognostic factors. Statistical analysis was performed using JMP 10 software (SAS Institute Inc., Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of CPNE5 and promoter methylation in ESCC cell lines. *CPNE5* mRNA expression levels differed among the 11 ESCC cell lines (Fig. 1A), and were lower compared with those of Het-1A cells, except for KYSE590 and KYSE1440. There were no significant differences in *CPNE5* mRNA expression levels between differentiated (0.00197±0.00172) and undifferentiated (0.00161±0.00133) cell lines (P=0.897). ESCC cell lines established from metastatic sites, including TT and TTn, and those established from lymph node metastases, including KYSE1170 and KYSE1260, expressed low levels of *CPNE5* mRNA. Western blot analysis using an anti-CPNE5 antibody illustrated high CPNE5 protein expression in an ESCC cell line with high *CPNE5* mRNA expression (KYSE590) and low CPNE5 protein expression in a low-expression ESCC cell line (TT) (Fig. 1B). Bisulfite sequencing analysis of *CPNE5* revealed that the CpG sites in the *CPNE5* DNA promotor region in all ESCC cell lines and Het-1A were completely methylated. The bisulfite sequencing results for Het-1A, KYSE590 and TT cells are presented as representative cell lines expressing high and low levels of *CPNE5* mRNA, respectively (Fig. 1C).

Characteristics of patients with ESCC. The median age of the 106 patients was 65 years (range, 44-84 years), and the female: Male ratio was 20:86. According to the UICC staging system (7th edition), 24, 29 and 53 patients were diagnosed with disease at pathological stages I, II and III, respectively. Adjuvant chemotherapy was administered to 36 patients (34%). The median duration of follow-up was 34.1 months, during which 44 patients (42%) experienced recurrence and 39 patients (37%) succumbed to the disease.

CPNE5 mRNA expression levels in clinical samples. The mean normalized *CPNE5* mRNA expression level was significantly lower in ESCC tissues (0.0107±0.0138) compared with the corresponding non-cancerous adjacent mucosal tissues (0.00829±0.0123; P=0.003; Fig. 2A).

Association between levels of CPNE5 mRNA and the clinicopathological characteristics of patients who underwent resection. There was no significant association between the low and high *CPNE5* expression groups with their clinicopathological characteristics (sex, tumor size and depth, lymphatic involvement, vascular invasion and pathological stage). By

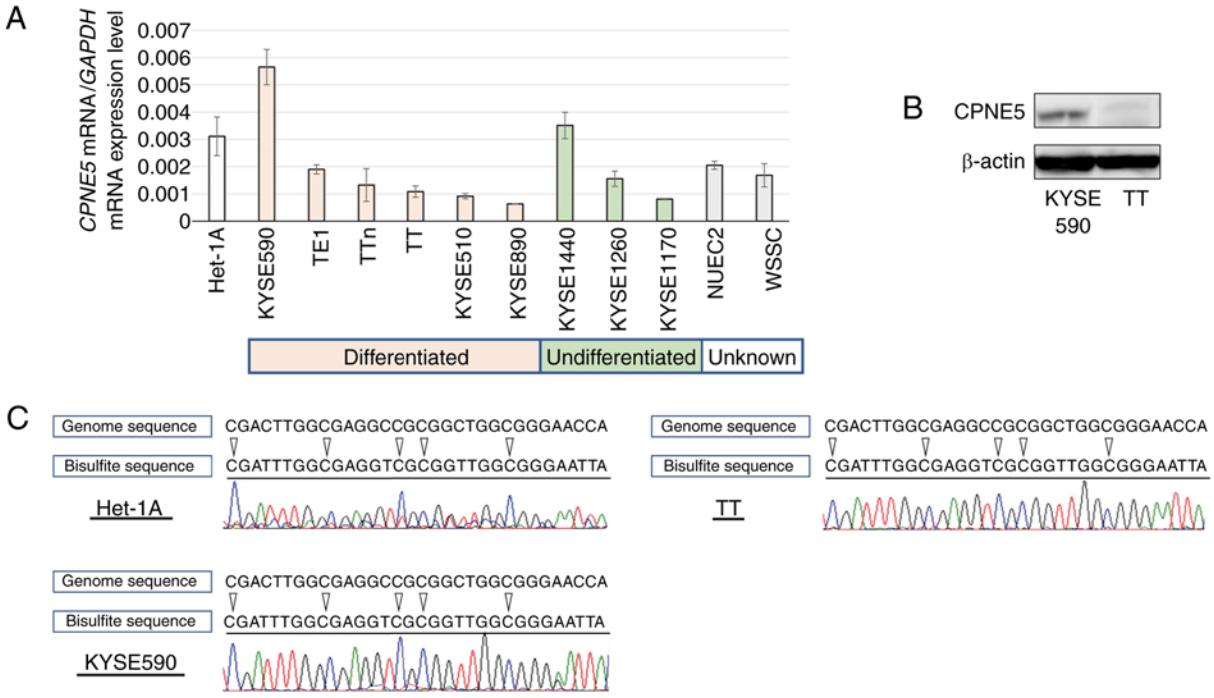

Figure 1. Analysis of *CPNE5* expression in cell lines. (A) *CPNE5* mRNA expression levels in a non-tumorigenic esophageal cell line (Het-1A) and esophageal carcinoma cell lines. (B) Western blot analysis of the CPNE5 protein expression level in ESCC cell lines. (C) Bisulfite sequencing analysis of the *CPNE5* promotor region in Het-1A cells and the esophageal cancer cell lines (KYSE590 and TT). The triangles above the sequencing results indicate the methylation sites. *CPNE5*, copine 5.

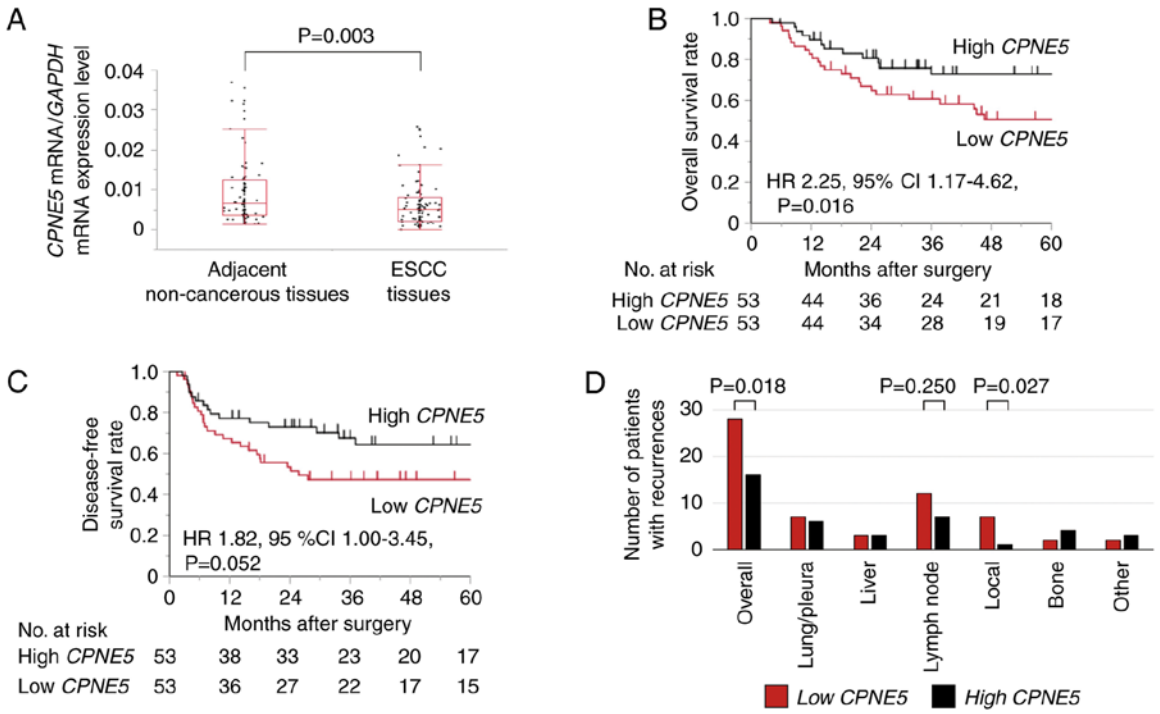

Figure 2. Analysis of *CPNE5* expression in clinical samples. (A) *CPNE5* mRNA expression levels in 106 resected ESCC tissues and adjacent non-cancerous esophageal mucosa. (B) Kaplan-Meier analysis of overall survival as a function of high or low expression of *CPNE5*. (C) Kaplan-Meier analysis of disease-free survival as a function of high or low expression of *CPNE5*. (D) Analysis of recurrence patterns. Numbers of sites of initial recurrence in the high and low *CPNE5* expression groups. ESCC, esophageal squamous cell carcinoma; *CPNE5*, copine 5; HR, hazard ratio; CI, confidence interval.

contrast, the percentage of patients who received adjuvant chemotherapy was significantly higher in the low *CPNE5* group (Table II).

Ability of CPNE5 mRNA expression levels to predict prognosis. Patients in the low *CPNE5* expression group experienced a significantly shorter OS time compared with

Table II. Association between the expression level of *CPNE5* mRNA and clinicopathological parameters in 106 patients with resected esophageal cancer.

Clinicopathological parameters	Low *CPNE5* in ESCC tissue, no. of patients	High *CPNE5* in ESCC tissue, no. of patients	P-value
Age, years			0.437
<65	23	28	
≥65	30	25	
Sex			1.000
Male	43	43	
Female	10	10	
Smoking history			0.487
Yes	39	43	
No	14	10	
Double cancer			0.698
Present	7	10	
Absent	46	43	
Tumor location			0.111
Ce	0	1	
Ut	3	5	
Mt	24	27	
Lt	25	15	
Ae	1	5	
Tumor multiplicity			1.000
Present	7	8	
Absent	46	45	
Tumor size, mm			0.151
<50	31	39	
≥50	22	14	
CEA, ng/ml			0.761
≤5	48	46	
>5	5	7	
SCC, IU/ml			0.531
≤1.5	36	33	
>1.5	15	19	
pT			0.116
T1 or 2	18	27	
T3	35	26	
Lymph node metastasis			0.843
Present	31	33	
Absent	22	20	
Differentiation			0.267
Differentiated	43	45	
Undifferentiated	10	5	
Lymphatic involvement			0.822
Present	39	41	
Absent	14	12	
Vascular invasion			1.000
Present	21	22	
Absent	32	31	
Intraepithelial progress			0.057
Present	18	22	
Absent	23	10	

Table II. Continued.

Clinicopathological parameters	Low *CPNE5* in ESCC tissue, no. of patients	High *CPNE5* in ESCC tissue, no. of patients	P-value
Pathological UICC stage			0.466
I	10	14	
II	17	12	
III	26	27	
Postoperative adjuvant chemotherapy			0.007
Present	25	11	
Absent	28	42	

CPNE5, copine 5; Ce, cervical esophagus; Ut, Upper thoracic esophagus; Mt, Middle thoracic esophagus; Lt, lower thoracic esophagus, Ae, abdominal esophagus; CEA, carcinoembryonic antigen; SCC, squamous cell carcinoma-related antigen; UICC, Union for International Cancer Control; pT, tumor depth.

Table III. Prognostic factors for overall survival of 106 patients.

	Univariate			Multivariable		
	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age, ≥65 years	1.45	0.75-2.79	0.259	-	-	-
Sex, male	2.19	0.94-6.43	0.072	-	-	-
Smoking	0.98	0.49-2.20	0.963	-	-	-
Double cancer	1.29	0.52-2.76	0.559	-	-	-
Tumor multiplicity	0.70	0.21-1.74	0.470	-	-	-
Tumor size, ≥50 mm	1.28	0.66-2.41	0.462	-	-	-
CEA, >5 ng/ml	1.34	0.50-2.97	0.528	-	-	-
SCC, >1.5 IU/ml	1.11	0.55-2.14	0.769	-	-	-
Tumor depth, pT3	2.75	1.38-5.96	0.003	1.48	0.72-3.27	0.293
Lymph node metastasis	2.36	1.19-5.09	0.013	1.53	0.64-3.92	0.349
Tumor differentiation, undifferentiated	2.75	1.22-5.62	0.002	2.10	0.92-4.37	0.075
Lymphatic involvement	8.61	2.63-53.0	<0.001	6.28	1.70-40.6	0.004[a]
Vascular invasion	1.39	0.73-2.61	0.304	-	-	-
Intraepithelial progress	0.64	0.32-1.27	0.204	-	-	-
Postoperative adjuvant chemotherapy	2.08	1.11-3.93	0.023	0.92	0.42-2.04	0.837
Low *CPNE5* expression	2.25	1.17-4.62	0.015	2.55	1.21-5.68	0.014[a]

[a]Statistically significant in multivariable analysis. CI, confidence interval; CEA, carcinoembryonic antigen; SCC, squamous cell carcinoma-related antigen; UICC, Union for International Cancer Control; *CPNE5*, copine 5.

those in the high *CPNE5* expression group (the 5-year OS rates were 55.6 and 77.3% for the low and high expression groups, respectively; P=0.016; Fig. 2B). Though the difference was not statistically significant, there was a similar trend observed in DFS (P=0.052; Fig. 2C). Univariate analysis revealed that tumor depth, lymph node metastasis, undifferentiated tumor phenotype, lymphatic involvement, postoperative chemotherapy and low *CPNE5* expression were significantly associated with lower survival rates. Multivariable analysis identified low *CPNE5* expression and lymphatic involvement as independent prognostic factors for OS (hazard ratio, 2.55; 95% confidence interval, 1.21-5.68; P=0.014; and hazard ratio, 6.28; 95% confidence interval, 1.70-40.6; P=0.004, respectively; Table III).

Analysis of patients with recurrence. Differences between recurrence patterns were predicted, since the Kaplan-Meier analysis revealed fold-differences between OS and DFS. Among patients with recurrence, a significant number were members of the low *CPNE5* group (P=0.018). Among patients with local recurrence, significantly more were members of the low *CPNE5* group (P=0.027; Fig. 2D). A similar tendency was observed in patients with lymph node recurrence (P=0.250; Fig. 2D). By contrast, the rates of distant

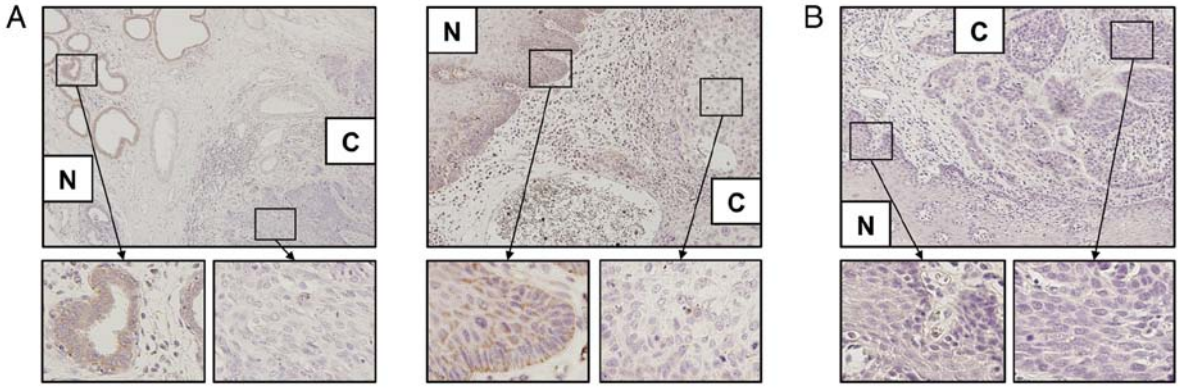

Figure 3. Immunohistochemistry analysis to detect the copine 5 protein expression levels in three representative patients (upper panels, x100 magnification; lower panels, x400 magnification). (A) Cancerous tissues exhibited reduced expression compared with adjacent non-cancerous tissues. (B) Cancerous tissue and non-cancerous tissue exhibited equivalent expression. N, non-cancerous tissue; C, cancerous tissue.

metastasis to tissues including the lung/pleura and liver were not significantly different between groups (Fig. 2D).

CPNE5 protein expression levels in clinical samples. The expression patterns of CPNE5 protein were evaluated using immunohistochemical staining. Among the 45 clinical samples, CPNE5 protein expression was suppressed in the cancerous tissue in 14 (31%) samples, equally expressed in cancerous and non-cancerous tissue in 19 (42%) samples and overexpressed in cancerous tissue in 12 (27%) samples. Representative examples of suppression in cancerous tissue, and an example of equivalent expression, are presented (Fig. 3).

Discussion

The results of the present study provided evidence to support the predictive value of *CPNE5* expression levels in ESCC tissues following curative resection. Specifically, the *CPNE5* mRNA expression levels of the majority ESCC cell lines were lower compared with those of a non-tumorigenic esophageal cell line. Bisulfite sequencing analysis was performed to reveal the mechanism of downregulation of *CPNE5* transcription, as the promoter region harbors CpG islands (25,26). Methylation of the *CPNE5* promoter region was detected in all ESCC cell lines and in a non-tumorigenic esophageal cell line, indicating that promoter hypermethylation did not contribute to the regulation of *CPNE5* transcription. Acetylation of histones (27), copy-number alterations (28), microRNAs (27) and genomic mutations (29) may therefore contribute to the downregulation of *CPNE5* transcription.

The expression levels of *CPNE5* mRNA in ESCC tissues were lower compared with those of adjacent non-cancerous tissue, revealing an association between *CPNE5* expression and the pathology of ESCC. *CPNE5* mRNA expression levels were not significantly associated with clinicopathological characteristics known to be associated with an unfavorable prognosis of ESCC [including tumor multiplicity, tumor size, tumor markers, lymphatic involvement, vascular invasion, and UICC stage (30)], although low levels of *CPNE5* mRNA were associated with shorter OS. Furthermore, multivariable analysis identified low levels of *CPNE5* mRNA as an independent risk factor of shorter OS. Therefore, *CPNE5* may serve as an effective marker for predicting prognosis compared with the tumor-node-metastasis classification of esophageal cancer.

CPNE5 mRNA expression levels were lower in patients who received adjuvant chemotherapy, although the administration of chemotherapy was at the physician's discretion. The association between adjuvant chemotherapy and *CPNE5* expression may be explained by the association, albeit not statistically significant, of *CPNE5* mRNA expression levels with tumor size and depth, in addition to intraepithelial progression, and physicians may therefore decide to administer adjuvant chemotherapy according to their interpretations of the totality of pathological findings. However, it was noted that patients in the low *CPNE5* expression group had poor prognoses despite adjuvant chemotherapy (31), suggesting an association between *CPNE5* expression and resistance to chemotherapy.

OS and DFS rates following curative resection of ESCC were lower in the low *CPNE5* group compared with the high *CPNE5* group. The difference in OS was statistically significant, although that for DFS was not. The Kaplan-Meier analysis demonstrated that the primary curves for DFS of the two groups overlapped. By contrast, the primary curves of OS were separated, and this difference may reflect the statistical difference. These results demonstrated that the low *CPNE5* group experienced shorter survival following recurrence. Accordingly, two hypotheses were developed to explain the data as follows: i) The clinicopathological findings indicate resistance to chemotherapy; thus, patients in the low *CPNE5* group may succumb following recurrence, as they did not benefit from chemotherapy; and ii) the differences in recurrence patterns may explain survival patterns; it was expected that recurrence in the low *CPNE5* group may arise in a site that is difficult to treat, for example the lung or liver, which is associated with poor prognosis following recurrence (32). The analysis of recurrence patterns, which was conducted to evaluate these hypotheses, revealed that significantly more patients in the low *CPNE5* group experienced more frequent recurrence compared with those in the high *CPNE5* group. In contrast to expectations, the difference in the numbers of patients in the low and high *CPNE5* groups was not significant for patients with lung and hepatic recurrences, which are associated with poor prognosis following recurrence.

However, it was noted that the differences in recurrence rates between the low and high *CPNE5* groups was explained by local and lymphatic recurrences, indicating that *CPNE5* expression may be associated with the local growth of esophageal cancer. To translate these findings into clinical practice, patients with low *CPNE5* expression ought to undergo surgery with rigorous lymph node dissection (33) and adequate surgical margins (34). Frequent follow-up, including esophagoscopy (35) and computed tomography (36), may enhance the detection of local recurrence.

There are certain limitations to the present study. First, an association between *CPNE5* expression and resistance to chemotherapy was identified. Unfortunately, it was not possible to obtain details of patients who received chemotherapy following recurrence. The analysis of biopsied or micro-dissected resected tissues from patients who have received neoadjuvant chemotherapy may help to investigate this further. Second, this was a retrospective study of a small number patients treated at a single center. External validation using large cohorts from multiple institutions is required to validate the present findings. Third, the function of *CPNE5* in esophageal cancer and its mechanism of regulation remain unexplained. Functional analysis of *CPNE5*-knockdown cell lines, protein expression and animal tumor xenograft models are required to overcome these limitations.

In conclusion, the present data suggested that *CPNE5* expression in ESCC tissues may represent a promising biomarker for predicting ESCC recurrence, particularly for patients with local recurrence, and may help ensure that patients receive optimal treatment and follow-up.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SU, MKa, TM and HT performed the experiments and the data analysis. SU, MKa, TM, HT, CT, DK, MKo, MS, MH, SY, GN and YK collected the cases and the clinical data. SU and MKa conceived and designed the study and prepared the initial manuscript. YK supervised the project. All authors contributed to the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects, and was approved by the Institutional Review Board of Nagoya University (Nagoya, Japan). Written informed consent for the use of clinical samples and data, as required by the institutional review board, was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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