Anti-phosphohistone H3 as an Independent Prognostic Factor in Human Esophageal Squamous Cell Carcinoma

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Abstract. *Background: The immunohistochemical staining of phospho-histone H3 (PHH3) has recently been reported to predict the prognosis of different tumors. However, it has not been evaluated in esophageal squamous cell carcinoma (ESCC). The aim of this study was to evaluate the prognostic impact of PHH3 in ESCC. Materials and Methods: The number of anti-phophohistone H3-positive nuclei [i.e. PHH3 mitotic index (MI)] was calculated by immunohistochemistry of 50 primary tumor samples obtained from patients with ESCC who underwent curative esophagectomy. Results: The PHH3 MI per 10 high-power fields ranged from 1 to 72 (median=15.5). When the patients were divided into two groups using a cut-off value of 10, the 5-year survival rate of the patients with PHH3 MI ≤10 was significantly higher than that of patients with PHH3 MI >10. Multivariate analysis indicated PHH3 MI to be an independent prognostic factor. Conclusion: The expression of PHH3 impacts the prognosis of patients with ESCC.*

Histone H3 is one of the five histone proteins which together form the major protein constituents of chromatin in eukaryotic cells. The mitosis marker anti-phosphohistone H3 (PHH3) was first introduced in 1997 (1). Histone H3 (Ser10) is phosphorylated in association with mitotic chromatin condensation in the late G_2 and M phases of the cell cycle (1, 2) but not during apoptosis (3). Therefore, PHH3 can be used as a specific mitotic marker. Expression of PHH3 has been investigated in several types of cancer (4-14). Among brain tumors, PHH3 staining was primarily found to support grading by facilitating mitotic counting, but it also had a prognostic value (4, 5). In cutaneous nodular melanoma, multivariate analysis indicated PHH3 to be a prognostic indicator (6). In gastric cancer, PHH3 overexpression was associated with histological type, vessel invasion, and lymph node metastasis, and it was found to be an independent prognostic factor (7). To our knowledge, the value of PHH3 expression in esophageal squamous cell carcinoma (ESCC) has not been previously evaluated.

The proliferation marker Ki-67 is expressed in all phases of the cell cycle (15). Several studies have assessed the proliferative activity of ESCC using the Ki-67 labeling index (LI) (16-18) and the correlation between PHH3 and Ki-67 expression has been studied in different carcinomas (7, 9, 13). However, the results have not been consistent. The cyclin-dependent kinase inhibitor p21 is a major negative regulator of the G_1 checkpoint (19). Many reports have suggested correlation between p21 expression and the prognosis of patients with ESCC (20, 21). However, there have been no reports regarding PHH3 and p21 expression.

In this context, the goals of the present study were to evaluate the prognostic impact of PHH3, compare the results with various clinicopathological parameters and with the Ki-67 and p21 LIs, and calculate an appropriate PHH3 mitotic index (PHH3 MI) cut-off value for predicting patient prognosis.

Materials and Methods

Patients and primary tissue samples. ESCC tumor samples were obtained from 50 patients with histologically-proven primary ESCC who underwent esophagectomy (potentially curative R0 resection) at the Kyoto Prefectural University of Medicine between 2000 and 2008. The samples were embedded in paraffin after 24 h of formalin fixation. Only patients who did not develop synchronous tumors or multiple metachronous tumors and who did not receive preoperative chemotherapy or radiation therapy met the eligibility criteria. All patients gave their written informed consent. Seventeen patients

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(34.0%) died of cancer recurrence. The median survival time was 14.4 months (ranging between 4 and 36 months). Thirty-three patients (66.0%) were still alive at the end of the study. The median follow-up period was 71.9 months (ranging between 36 and 140 months). Staging was principally based on the International Union Against Cancer (UICC)/TNM Classification of Malignant Tumors (7th edition) (22).

Immunohistochemistry. Paraffin sections (3-μm thick) of tumor tissue were subjected to immunohistochemical staining for PHH3, Ki-67 and p21 using the avidin-biotin-peroxidase method. Briefly, paraffin sections were de-waxed in xylene and dehydrated through a graded series of alcohols. Antigen retrieval was performed by heating the samples in Dako REAL Target Retrieval Solution (Glostrup, Denmark) for 40 min at 95˚C. Endogenous peroxidase activity was quenched by incubating the sections for 30 min in 0.3% H_2O_2. The sections were incubated for 1 h at room temperature with the following phosphohistone H3 (Millipore, Billerica, MA, USA), Ki-67 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and p21 (Cell Signaling Technology, Beverly, MA, USA) antibodies. The avidin-biotin-peroxidase complex system (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) was used for color development with diaminobenzidine tetrahydrochloride. The sections were counterstained with hematoxylin. Finally, the sections were dehydrated through a graded series of alcohols, cleared in xylene, and mounted. Control sections of known positive ESCC were included in each antibody run, and negative control sections were produced by omitting the primary antibody.

Determination of proliferative activity by immunohistochemistry was performed quantitatively by counting immunoreactive tumor cells. PHH3 MI was calculated as the number of positive cells in 10 consecutive high-power fields (HPFs) (×400) in the areas with the highest mitotic activity. Only distinct immunoreactive tumor cell nuclei were counted. Ki-67- and p21-stained cells were quantified in five selected fields of the highest proliferative activity at ×400 magnification. The LI of each case was calculated as the number of positive cells divided by the total number of examined cells in all the examined fields.

Statistical analysis. Statistical analyses were carried out using the Student's *t*-test for comparisons between two groups, the Tukey-Kramer HSD test for multiple comparisons, or Fisher's exact test to investigate the correlations between clinicopathological parameters and PHH3 MI. Survival curves were constructed using the Kaplan-Meier method, and differences in survival were examined using the log-rank test. Multivariate analysis of the factors influencing survival was performed using the Cox proportional hazard model. Differences were considered significant when the associated *p*-value was less than 0.05. All analyses were performed using statistical software (JMP, version 10; SAS Institute Inc., Cary, NC, USA). Correlation analyses were performed by creating Fit Y by X plots using JMP.

Results

PHH3. An immunohistochemical examination of non-cancerous esophageal epithelia performed with PHH3 antibody demonstrated that PHH3-positive cells were loosely scattered in the parabasal cell layer. PHH3- positive tumor cells were clearly identified by their brown nuclear staining (Figure 1A and B). The number of PHH3-positive figures ranged from 1 to 72 [median=15.5; mean±standard deviation (SD): 18.2±14.5]. Photographs of well- and poorly-differentiated ESCC tumor samples with high and low PHH3 MI are shown in Figure 1C.

The correlations between PHH3 MI and various clinicopathological parameters were analyzed. The mean PHH3 MI increased from 15.4 in well-differentiated SCC to 23.8 in poorly-differentiated SCC. However, the correlation between histological type and PHH3 MI failed to achieve statistical significance. No correlation was found between the PHH3 MI and other clinicopathological features (Table I). To evaluate the association between PHH3, Ki-67, and p21 expression, the immunohistochemical analysis of Ki-67 and p21 was performed. The proportion of Ki-67- and p21-positive tumor cells varied widely between the tumors. The minimum Ki-67 LI was 5.3%, and the maximum was 56.3% (median=30.2%; mean±SD=30.6%±12.8). The minimum p21 LI was 2.0%, and the maximum was 54.8% (median=26.8%, mean±SD=24.8%±12.2). There were no correlations between PHH3 MI and Ki-67 LI (R^2=0.004, p=0.653), nor between PHH3 MI and p21 LI (R^2=0.054, p=0.105) (Figure 2A and B).

Next, we analyzed the effect of the PHH3 MI on the survival of patients with ESCC. To determine an appropriate PHH3 MI cut-off value for predicting the survival of ESCC patients, we analyzed the 5-year survival rate according to various cut-off values. When the patients were divided into two groups using a PHH3 MI cut-off value of 10, the 5-year survival rate of the patients with PHH3 MI ≤10 was 88.9%, which was significantly higher than that of the patients with PHH3 MI >10 (47.1%) (Figure 2C). Therefore, we decided that 10 was an appropriate cut-off PHH3 MI value for predicting survival and performed further analyses using this value. We divided the patients into two groups, PHH3 MI ≤10 (n=18) and PHH3 MI >10 (n=32), and compared their clinicopathological features. We found that the percentage of differentiated SCC was significantly higher among the patients with PHH3 MI ≤10 (94.4%) than among the patients with PHH3 MI >10 (59.4%) (Table II). No correlation was found between PHH3 MI and any other clinicopathological parameter (Table II). Moreover, there were no associations between PHH3 MI, Ki-67, or p21 (Table II). We also assessed which of the nine factors (age, gender, pT, pN categories, pStage, histological type, lymphatic invasion, venous invasion and PHH3 MI (cut-off value=10) influenced survival after the curative resection of esophageal cancer. In univariate analysis of survival after esophagectomy, age, pT, pN, lymphatic invasion and PHH3 MI were found to be prognostic factors (p=0.0495, 0.024, 0.022, 0.021 and 0.015, respectively) (Table III). Multivariate analysis demonstrated that pN and PHH3 MI were independent prognostic factors (p=0.041 and 0.008, respectively) (Table IV). These findings

Table I. *Associations between the clinicopathological features of esophageal cancer and phospho-histone H3 mitotic index (PHH3 MI).*

Variable	Number of cases	PHH3 MI	*p*-Value
Age			
<60 years	17	17.2±8.1	0.734
≥60 years	33	18.7±17.0	
Gender			
Male	42	19.0±14.8	0.340
Female	8	13.6±12.7	
pT			
pT1	22	22.4±17.1	n.s.
pT2	6	16.5±7.9	
pT3	22	14.4±12.2	
pN			
pN0	20	16.1±13.2	n.s.
pN1	16	19.3±17.4	
pN2	12	18.9±13.9	
pN3	2	25.5±9.2	
pStage			
I	15	19.0±13.6	n.s.
II	15	18.5±18.5	
III	20	17.3±12.4	
Histological type			
Well-differentiated SCC	14	15.4±13.2	n.s.
Moderately-differentiated SCC	22	16.4±15.7	
Poorly-differentiated SCC	14	23.8±13.1	
Lymphatic invasion			
Negative	20	18.5±13.7	0.894
Positive	30	17.9±15.2	
Venous invasion			
Negative	25	20.2±13.5	0.325
Positive	25	16.1±15.4	

Mean±SD. Student's *t*-test was used for two-group comparisons and the Tukey-Kramer HSD test for multiple comparisons. pT: Pathological T stage, pN: pathological N stage, pStage: pathological stage, SCC: squamous cell carcinoma, n.s.: not significant in multiple comparisons.

Table II. *Associations between the clinicopathological features of esophageal cancer and phospho-histone H3 mitotic index (PHH3 MI) (cut-off value=10).*

Variable	PHH3		*p*-Value
	≤10 (n=18)	>10 (n=32)	
Age			
<60 years	4	13	0.227
≥60 years	14	19	
Gender			
Male	14	28	0.436
Female	4	4	
pT			
pT1	7	15	0.768.
pT2-3	11	17	
pN			
pN0	9	11	0.370
pN1-3	9	21	
pStage			
I	5	10	1.000
II-III	13	22	
Histological type			
Differentiated SCC	17	19	0.009*
Poorly differentiated SCC	1	13	
Lymphatic invasion			
Negative	8	12	0.765
Positive	10	20	
Venous invasion			
Negative	7	18	0.377
Positive	11	14	
Ki-67 LI+	29.3±3.03	31.3±2.27	0.587
p21 LI+	26.7±2.89	23.7±2.17	0.404

+Mean±SD. Student's *t*-test was used for two-group comparisons. *$p<0.05$: Fisher's exact test.

suggested that the PHH3 MI is a strong independent prognostic factor for patients with ESCC, with these patients having a PHH3 MI >10 having a poorer prognosis.

Discussion

To our knowledge, this is the first report regarding PHH3 MI and ESCC tissues. There have been other reports about the clinicopathological role of PHH3 MI in several types of cancers (6-12), but their results were inconclusive. In our study, PHH3 MI was correlated with histological type, but not with other clinicopathological features. The mean PHH3 MI in well-differentiated SCC was lower than that in poorly- differentiated SCC. Furthermore, the percentage of differentiated SCC was significantly higher among the patients with PHH3 MI ≤10 than among the patients with PHH3 MI >10. Goodarzi *et al*. found PHH3 to be a potential supportive marker along with histology in differentiating low-grade dysplasia from indefinite for dysplasia and high-grade dysplasia from adenocarcinoma in 88 endoscopic biopsy samples of Barrett's esophagus-associated neoplastic lesions (8). Aune *et al*. demonstrated that PHH3 expression was significantly higher in serous ovarian carcinomas than that in non-serous ovarian carcinomas (9). Takahashi *et al*. showed that correlations with PHH3 overexpression were noted for histological type, vessel invasion and lymph node metastasis in gastric cancer (7). Ladstein *et al*. and others reported that PHH3 expression was associated with tumor thickness and presence of ulceration in cutaneous melanoma and that it was significantly higher in melanomas than in benign nevi (6, 10, 11). Williams *et al*. found that PHH3 expression was significantly correlated with the grade of estrogen receptor-positive breast cancer (12). Their and our results suggest that PHH3 MI is closely linked with the differentiation of cancer cells and is related to the invasive ability of tumors.

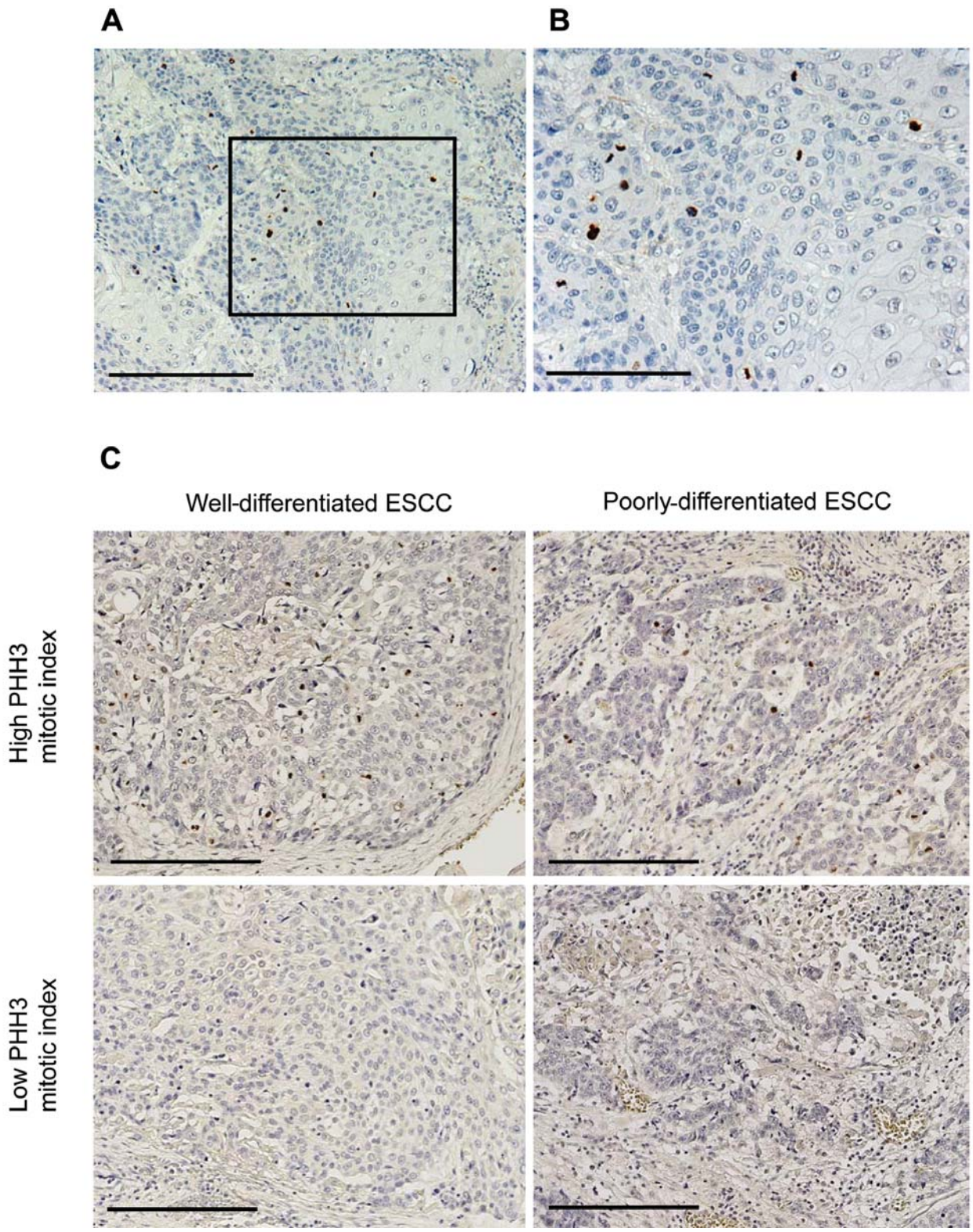

Figure 1. *A: Immunohistochemical staining of primary human esophageal squamous cell carcinoma (ESCC) samples with phospho-histone H3 (PHH3) antibody (×200). Bar=200 μm. B: Magnification of the inset shown in 1A (×400). Bar=100 μm. C: Immunohistochemical staining of well- and poorly-differentiated ESCC tumor samples with high and low PHH3 mitotic index (MI). Well-differentiated ESCC with PHH3 MI=72 (high) and with PHH3 MI=1 (low); poorly differentiated ESCC with PHH3 MI=39 (high) and with PHH3 MI=11 (low) (×200). Bar=200 μm.*

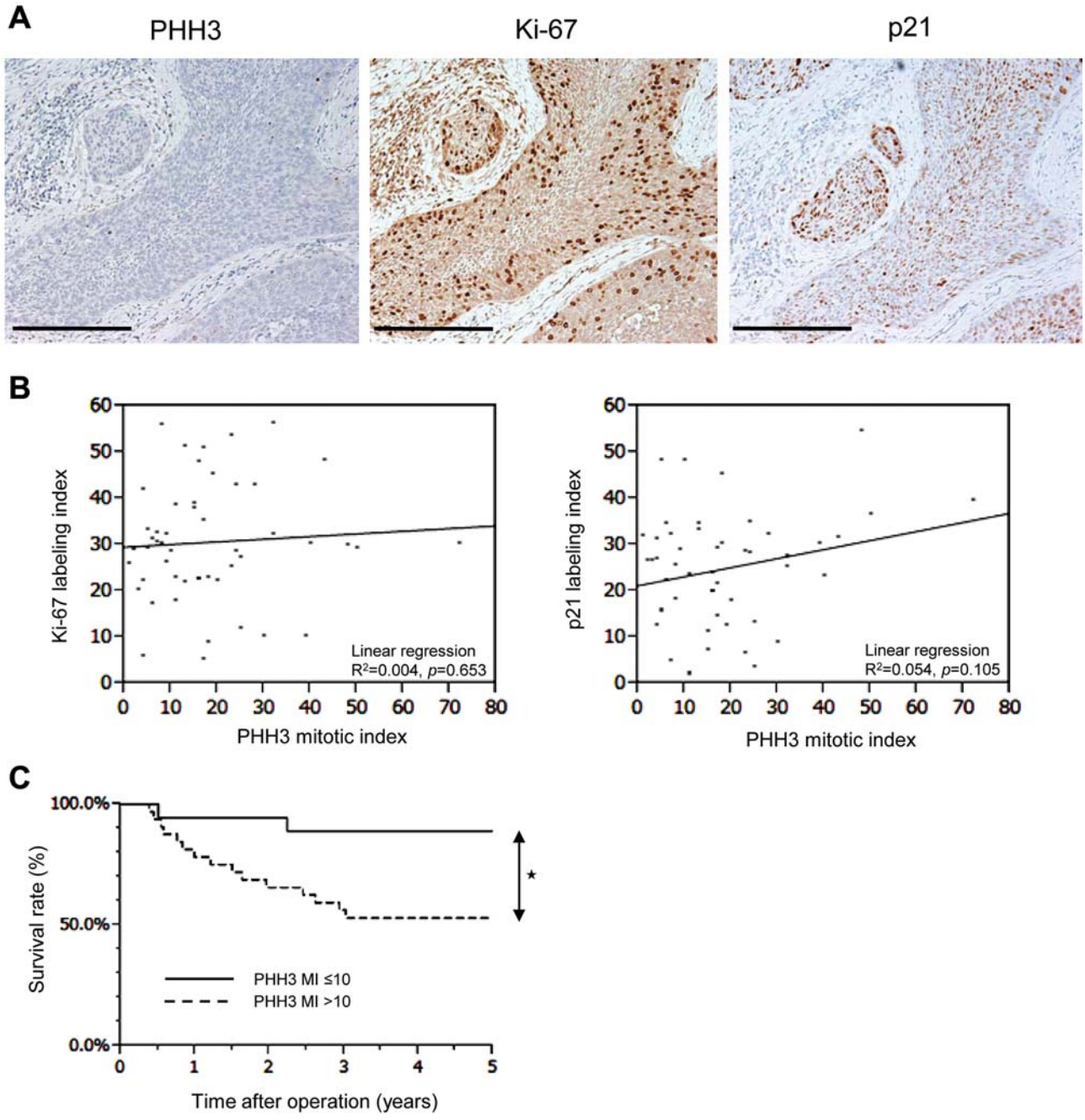

Figure 2. *A: Immunohistochemical staining of primary human esophageal squamous cell carcinoma (ESCC) samples with phospho-histone H3 (PHH3), Ki-67 and p21 antibodies (×200). Bar=200 μm. B: Scatter plots with regression lines illustrating the relationships between PHH3 mitotic index (MI), Ki-67 labeling index (LI), and p21 LI. C: Survival curve of patients with ESCC using the Kaplan-Meier method. The patients were classified into two groups, PHH3 MI ≤10 (n=18) and PHH3 MI >10 (n=32). Statistical analysis: log-rank test, *p<0.05.*

The prognostic significance of the PHH3 MI has been reported with different cut-off values for several types of carcinomas. We showed that the median and mean PHH3 MI per 10 HPFs were 15.5 and 18.2, respectively. In addition, we found that PHH3 MI was an independent prognostic factor for ESCC with a cut-off value of 10. Kim *et al*. demonstrated that the mean PHH3 MI per 10 HPFs was 3.2 and that the PHH3 MI of ≥6 per 10 HPFs was the most appropriate prognostic cut-off value for the prediction of recurrence-free survival of patients with meningioma (4). Aune *et al*. showed

Table III. *The 5-year survival rate of patients with esophageal cancer according to various clinicopathological parameters.*

Variable	5-Year survival rate (%)	*p*-Value
Age		
<60 years	47.1	0.0495*
≥60 years	75.8	
Gender		
Male	66.6	0.915
Female	62.5	
pT		
pT1	81.8	0.024*
pT2-3	53.6	
pN		
pN0	85.0	0.022*
pN1-3	53.3	
pStage		
I	80.0	0.175
II-III	60.0	
Histological type		
Differentiated SCC	69.4	0.400
Poorly-differentiated SCC	57.1	
Lymphatic invasion		
Negative	85.0	0.021*
Positive	53.1	
Venous invasion		
Negative	76.0	0.126
Positive	56.0	
PHH3 MI		
≤10	88.9	0.015*
>10	47.1	

*$p<0.05$: Log-rank test. PHH3 MI: phospho-histone H3 mitotic index, SCC: squamous cell carcinoma.

Table IV. *Prognostic factors of esophageal cancer according to multivariate analysis.*

Variable	Risk ratio	95% CI	*p*-Value
Age			
<60 years	1.591		
≥60 years		0.582-4.418	0.362
pT			
pT1	2.950		
pT2-3		0.988-10.839	0.053
pN			
pN0	3.305		
pN1-3		1.049-14.559	0.041*
Lymphatic invasion			
Negative	2.908		
Positive		0.909-12.897	0.074
PHH3 MI			
≤10	5.427		
>10		1.489-34.893	0.008*

95% CI: 95% Confidence interval; *$p<0.05$: Cox's proportional hazards model; PHH3 MI: phospho-histone H3 mitotic index.

that the median PHH3 MI per 10 HPFs was 48.5 and that high expression of PHH3 had a negative impact on the survival of patients with ovarian carcinoma (9). Takahashi *et al.* showed that the median PHH3 MI per approximately 1000 cancer cells was 0.2, and when patients with gastric cancer were divided into two groups using a cut-off value of 0.9 per approximately 1000 cancer cells (at the higher quartile), the high PHH3-expressing group had a worse prognosis than the low PHH3-expressing group (7). PHH3 MI varied according to the type of carcinoma, and the ranges used to count PHH3-positive cells were not consistent; we decided that a count of 10 per 10 HPFs was an appropriate cut-off value to predict survival in ESCC. Our results suggest that the level of PHH3 expression impacts the prognosis of ESCC patients at this clinically useful cut-off value.

Several studies have assessed the correlation between Ki-67 and PHH3 expression in various carcinomas. In our study, PHH3 MI was not correlated with Ki-67 LI. Takahashi *et al.* found that there was no correlation between PHH3 and Ki-67 expression in gastric cancer. While PHH3 expression was found to be an independent factor for poor prognosis, Ki-67 expression did not influence prognosis (7). Brenner *et al.* reported PHH3 to be a better mitotic marker than Ki-67 in endometrial tissues (13). Ki-67 is commonly used to assess cell proliferation; this factor reacts with a nuclear antigen present throughout the cell cycle (late G_1, S, G_2, and M phase) of proliferating cells but is absent from quiescent (G_0) cells (23). However, PHH3 is expressed in the late G_2 and M phases of the cell cycle. The different rates of progress through each phase of the cell cycle may explain why no correlation was found between PHH3 and Ki-67 expression, neither in previous studies nor ours. Furthermore, the process of counting PHH3-positive cells is simpler and faster than that for counting Ki-67-positive cells. Thus, we consider PHH3 MI to be a suitable proliferation marker for clinical practice.

The *p21* gene acts as a major regulator of the G_1 checkpoint by binding to and inhibiting the activities of most cyclin (CDK) complexes (19), and some studies have reported that p21 appeared to exhibit oncogenic activities in ESCC (24, 25). However, in the present study, no correlation was found between PHH3 and p21 expression. One of the reasons for this observation may be due to their contrasting roles in the phases of the cell cycle, although further examinations are necessary. As far as we know, this is the first report investigating the relationship between PHH3 and p21 expression in clinical samples of cancer.

PHH3 MI is correlated with histological type and can be used as an independent prognostic factor for patients with ESCC, by selecting an adequate cut-off value.

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