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Expression of Minichromosome Maintenance Protein 7 and Smad 4 in Squamous Cell Carcinoma of the Esophagus

Ji Hyun Ahn · Hee Kyung Chang

Department of Pathology, Kosin University College of Medicine, Busan, Korea

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Corresponding Author
Hee Kyung Chang, M.D.
Department of Pathology, Kosin University College of Medicine, 34 Amnam-dong, Seo-gu, Busan 602-702, Korea
Tel: 82-51-990-6323
Fax: 82-51-241-7420
E-mail: changhkg@ns.kosinmed.or.kr

Background : Minichromosome maintenance protein 7 (MCM 7) performs a direct role in the initiation of DNA replication, which suggests that it may prove useful as a marker of cell proliferation. *Smad 4* is a tumor suppressor gene that mediates the transforming growth factor β pathway. The principal objective of this study was to characterize the expression of MCM 7 and Smad 4 and to analyze their relationship to clinicopathological parameters in patients with esophageal squamous cell carcinoma. **Methods :** Expression levels of MCM 7 and Smad 4 were evaluated via immunohistochemistry on formalin-fixed and paraffin-embedded tissues from 67 cases of esophageal squamous cell carcinoma. **Results :** High levels of MCM 7 expression were detected in 53 cases (74.6%), and were associated with higher T stages ($p = 0.030$). Kaplan-Meier survival curves demonstrated that patients with higher levels of MCM 7 expression had poorer prognoses, although this association was not significant ($p = 0.086$). Loss of Smad 4 expression was noted in 18 cases (23.4%), and was not associated with clinicopathological characteristics, including MCM 7 expression, or prognosis. **Conclusions :** MCM 7 expression is associated with the invasiveness of esophageal squamous cell carcinoma. Altered expression of Smad 4 does not appear to have pathobiological significance in esophageal carcinoma.

Key Words : MCM protein 7, human; Smad 4 protein, human; Carcinoma, squamous cell; Esophageal neoplasms

Esophageal cancer is the 9th most common cancer in Korean males.[1] Squamous cell carcinoma is the most common histological type worldwide, including in Korea. Risk factors thus far identified are cigarettes, alcohol, lack of vitamin A, C, and E, lack of folic acid, a history of achalasia, corrosive esophagostenosis, hyperkeratosis, Plummer-Vinson syndrome, and head and neck tumors.[2-4] The esophagus lacks a serosal layer, although the lymphatic channels are well-developed, and early-stage esophageal cancer spreads readily into the adjacent organs, thus resulting in an overall poor prognosis. This poor prognosis issue persists, despite recent improvements in surgical methods and therapeutic agents.[5] Although several previous studies have focused on the identification of prognostic molecular markers in this cancer, no significant prognostic markers have yet been identified.

The minichromosome maintenance (MCM) complex is a DNA-binding heterohexamer complex formed by minichromosome maintenance proteins 2-7 (MCM 2-7). The MCM complex functions as a "licensing factor," which allows the initiation of DNA replication. During cell division, it allows only one DNA replication per cycle. The MCM complex combines with the origin recognition complex, Cdc 6, and Cdt 1 to form the prereplicative complex (pre-RC).[6-10] Once the pre-RC is built up, the initiation of DNA replication is permitted. During DNA synthesis, the MCM complex dissociates from the replication origin, thereby preventing replication from occurring until the cell achieves the G1 phase of the next cycle.[6-10] MCM proteins are thought to be useful markers for proliferation, as it has been demonstrated in replicating but not quiescent cells.[11-13] Additionally, MCM proteins are utilized as markers for the diagnosis of invasive cancer and carcinoma *in situ*. Recent studies have demonstrated an increase in MCM proteins in malignant tumors such as adenocarcinoma of the lung, colon, and prostate, papillary thyroid carcinoma and endometrial carcinoma.[11-16] A few studies have also evaluated the expression of MCM 2, 4, and 5 in esophageal squamous cell carcinoma, but, to the best of our knowledge, no research into MCM 7 expression in esophageal carcinoma has been reported thus far in the English-language literature.[17-19]

The transforming growth factor β (TGF-β)/Smad pathway is

generally considered a proliferation control mechanism of normal epithelial, endothelial, and hematopoietic cells.[20-23] Smad 4 mediates cell cycle arrest in response to TGF-β in the G1 phase, and seems to hinder recruitment and activation of MCM proteins in an indirect manner. Smad 4-mediated TGF-β signaling downregulates cyclin E-cdk2 kinase that is required for Cdc 6 phosphorylation to be able to load MCM proteins.[24,25] Smad 4 suppression has been observed in cancers of the pancreas, head and neck, colon, stomach and esophagus.[20-23,26-28] Therefore, we speculated that the expression of Smad 4 is opposite to that of MCM 7 in malignancy.

The principal objective of this study was to determine the prognostic significance of MCM 7 and Smad 4 expression in esophageal squamous cell carcinoma in Korea. To achieve this objective, we evaluated expression of MCM 7 and Smad 4 in esophageal squamous cell carcinoma via immunohistochemistry, and assessed the relationship between their expression and relevant clinicopathological parameters.

MATERIALS AND METHODS

Materials

Surgical specimens were acquired from 67 patients with esophageal squamous cell carcinoma who had undergone surgery at the Kosin University Gospel Hospital between August 1997 and November 2002. Both neoplastic and non-neoplastic tissues were obtained from each specimen. They were fixed with formalin and embedded in paraffin.

Analysis of clinicopathological characteristics

Patient data including sex, age, and survival period were extracted from the medical records of 67 patients. Hematoxylin and eosin-stained slides were reviewed in each case to assess pathologic parameters, including histologic grade, invasion depth, and presence of lymph node metastasis, lymphatic invasion, vascular invasion, and neural invasion. The stage of each squamous cell carcinoma was determined in accordance with the staging schemata of the 2009 American Joint Committee on Cancer tumor, node, metastasis (TNM) staging.

Immunohistochemical staining

Paraffin-embedded tissue sections were cut to a 5 μm thickness, mounted on positively charged slides, deparaffinized in xylene for 10 minutes, and rehydrated through a graded series of ethyl alcohol concentrations. Antigen retrieval was conducted at 750 W for 45 minutes in citrate buffer (pH 6.0). MCM 7 (1 : 200, LabVision Neomarkers, Fremont, CA, USA) and Smad 4 (1 : 100, Abcam, Cambridge, UK) were used as a primary antibody. After the application of primary antibody, sections were incubated for 60 minutes at room temperature. After washing in tris-buffered saline, secondary antibody was applied for 15 minutes. The sections were washed again and incubated for 15 minutes with peroxidase-labeled streptavidin reagent. To visualize the bound immune complex, 3-amino-9-ethyl carbazole was utilized as a chromogen and nuclear counterstaining was conducted with Mayer's hematoxylin.

Interpretation of immunohistochemistry

Distinct nuclear staining of MCM 7 was considered positive. The percentage of MCM 7 was determined by counting the number of positive cells. At least 200 cells were counted in the most frequently labeled areas. Counts were performed in high-power fields ($\times$ 400). Samples were classified into 4 groups according to the percentage of MCM 7-positive tumor cells: 1 (0-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%). For statistical analysis, those with low (group 1) and high (group 2-4) MCM 7 expression were compared. In order to evaluate the expression of Smad 4, tumor cells were compared with normal epithelium. Cytoplasmic staining in tumor cells that was stronger than the staining in normal epithelium was considered positive. Tumors were classified on the basis of Smad 4 expression. The cases with Smad 4 expression in more than 50% of tumor cells were classified as Smad 4-positive. Tonsil and lung adenocarcinoma were used as controls for MCM 7- and Smad 4-positive staining.

Statistical analysis

SPSS ver. 17.0 (K for window, SPSS Inc., Chicago, IL, USA) was used for statistical analysis, and p-values less than 0.05 were considered statistically significant. Cross-tabulation analysis using Pearson's χ^2 tests was conducted to determine the relationship between the expression of MCM 7 and Smad 4 and the clinicopathological characteristics, with the exception of the survival period. Kaplan-Meier analysis using log-rank tests was conducted to estimate cumulative disease-free survival rates.

RESULTS

Clinicopathological characteristics

The mean age of the 67 patients was 61 years (range, 41 to 75 years) and the sex ratio was 10.2 : 1 (61 males : 6 females). Mean survival time was 44 months (range, 5 to 106 months) during the follow-up period. The most common site of cancer was mid-esophageal (53 cases), whereas the most frequent histologic grade was moderately differentiated (32 cases). A total of 16 cases were classified as T1 stage tumors, 16 as T2, 29 as T3, and 6 as T4. Two cases had distant metastases. Lymph node metastasis was observed in 28 cases, and lymphatic invasion in 19 cases. Neural invasion and vascular invasion were noted in 7 and 1 cases, respectively.

MCM 7 expression

In the normal epithelium, MCM 7 showed nuclear staining in basal and parabasal cells (Fig. 1A). Fifty of 67 cases (74.6%) exhibited distinct nuclear staining in 26% or more cells (Fig. 1B, C). MCM 7 expression increased with increasing T stage, and the observed difference was significant ($p = 0.030$). We noted no significant relationship between MCM 7 expression and histologic grade, location, lymph node metastasis, distant metastasis, lymphovascular, or neural invasion (Table 1). The five-year survival rate determined by the Kaplan-Meier method was 39.2% and 56.0% in the high and low MCM 7 groups, respectively, but this difference was not significant ($p = 0.086$) (Fig. 2).

Smad 4 expression

Smad 4 was detected in the cytoplasm of normal epithelial cells (Fig. 1A) and tumor cell nests (Fig. 2). Eighteen of 67 cases (23.4%) showed a loss in Smad 4 expression over more than 50% of the tumor volume (Fig. 3). Smad 4 expression, however, was not correlated with clinicopathological factors such as histologic grade, location, invasion depth, nodal and distant metastasis, neural invasion, and lymphovascular inva-

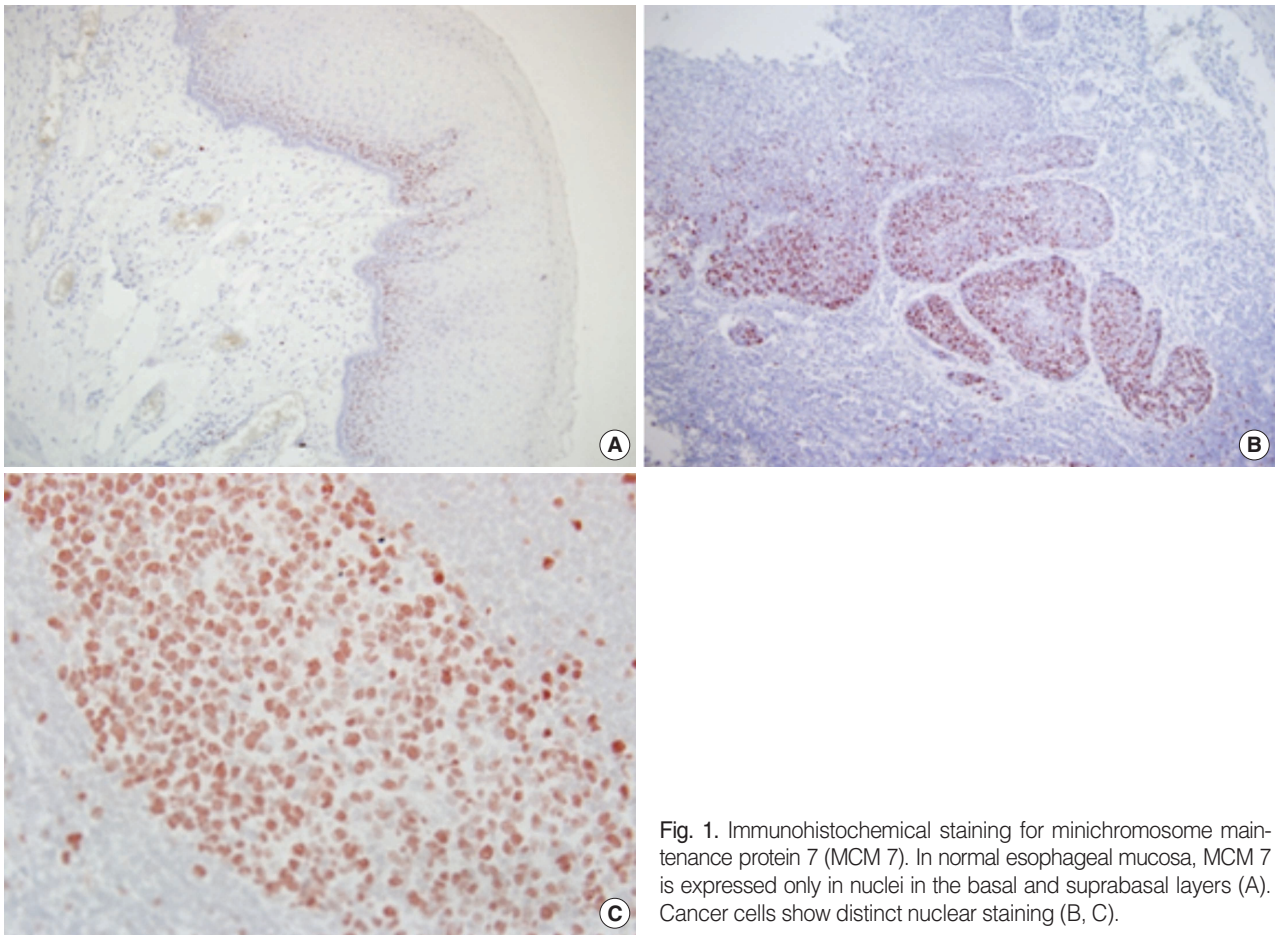

Fig. 1. Immunohistochemical staining for minichromosome maintenance protein 7 (MCM 7). In normal esophageal mucosa, MCM 7 is expressed only in nuclei in the basal and suprabasal layers (A). Cancer cells show distinct nuclear staining (B, C).

Table 1. Relationship between expression of minichromosome maintenance protein 7 (MCM 7) and clinicopathological parameters

Parameters	n	Low MCM 7 (%) (n = 17)	High MCM 7 (%) (n = 50)	p-value[a]
T stage				0.030
I	16	7 (43.8)	9 (56.3)	
II	16	4 (18.8)	12 (81.3)	
III	29	6 (20.7)	23 (79.3)	
IV	6	0 (0.0)	6 (100.0)	
Node metastasis				0.533
Absent	39	11 (28.2)	28 (71.8)	
Present	28	6 (21.4)	22 (78.6)	
Distant metastasis				0.406
Absent	65	17 (26.2)	48 (73.8)	
Present	2	0 (0.0)	2 (100.0)	
Histologic grade				0.370
Well differentiation	22	3 (13.6)	19 (86.4)	
Moderate differentiation	32	11 (34.4)	21 (65.6)	
Poor differentiation	13	3 (23.1)	10 (76.9)	
Location				0.765
Upper third	2	0 (0.0)	2 (100.0)	
Mid third	53	14 (26.4)	39 (73.6)	
Lower third	12	3 (25.0)	9 (75.0)	
Neural invasion				0.480
Absent	60	16 (26.7)	44 (73.3)	
Present	7	1 (14.3)	6 (85.7)	
Vascular invasion				0.560
Absent	66	17 (25.8)	49 (74.2)	
Present	1	0 (0.0)	1 (100.0)	
Lymphatic invasion				0.612
Absent	48	13 (27.1)	35 (72.9)	
Present	19	4 (21.1)	15 (78.9)	

[a]Significance level of the chi-square test.

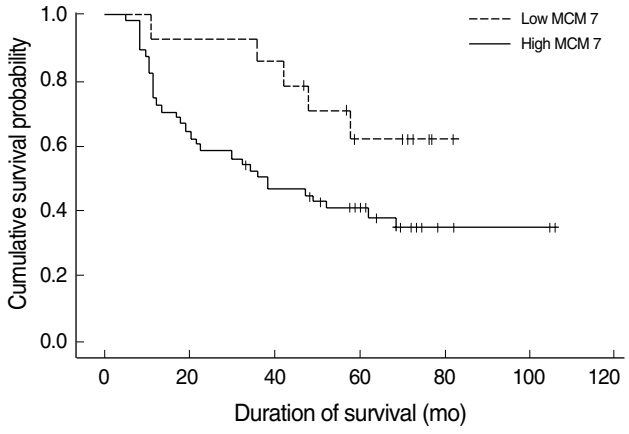

Fig. 2. Kaplan-Meier analysis of the overall survival of 67 patients with esophageal squamous cell carcinoma according to minichromosome maintenance protein 7 (MCM 7) expression status. Patients with tumors showing higher MCM 7 expression levels exhibit slightly reduced overall survival as compared to patients with tumors that are MCM 7 negative (log-rank test, p = 0.086).

sion (Table 2). We noted no significant survival difference between the Smad-positive and Smad-negative groups, which had, respectively, 5-year survival rates of 43.9% and 41.8% (Fig. 4).

The relationship of MCM 7 and Smad 4 expression

Five (29.4%) and 13 (26.0%) cases, respectively, showed loss of Smad 4 expression in low and high MCM 7 expression groups (p = 0.786), suggesting that there was no association between these two markers.

DISCUSSION

In this study, we evaluated MCM 7 and Smad 4 expression in esophageal squamous cell carcinoma. The frequency of higher MCM 7 expression increased with increasing T stage (p = 0.030). However, we were unable to demonstrate a relationship between MCM 7 expression and clinicopathological characteristics including histologic grade, lymph node metastasis, distant metastasis, lymphovascular, and neural invasion. Five-year survival tended to be increased in the low MCM 7 expression group, but this

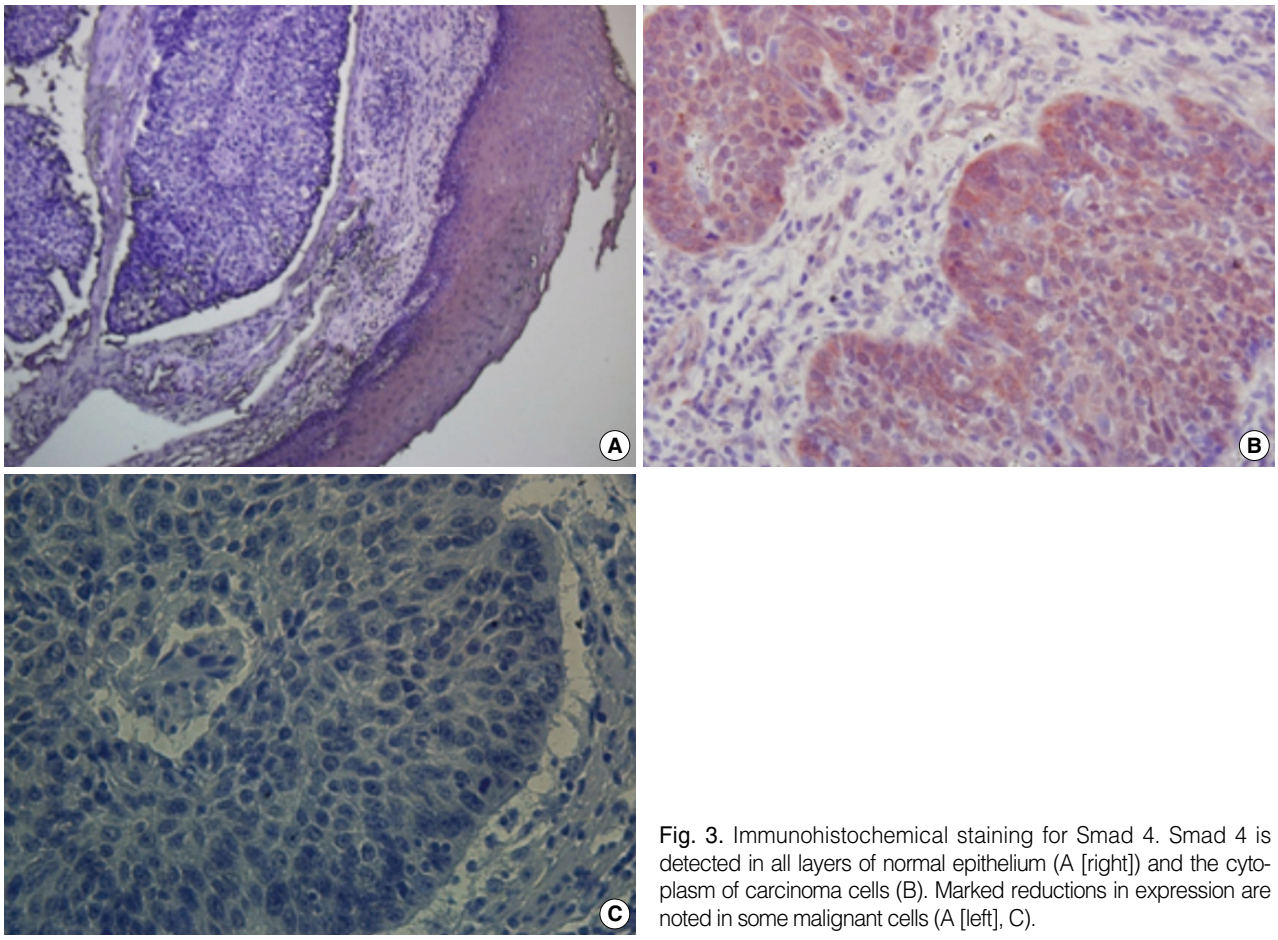

Fig. 3. Immunohistochemical staining for Smad 4. Smad 4 is detected in all layers of normal epithelium (A [right]) and the cytoplasm of carcinoma cells (B). Marked reductions in expression are noted in some malignant cells (A [left], C).

was not significant (p = 0.086). A loss of Smad 4 expression was detected in 18 of 67 cases (23.4%), and there was no correlation between Smad 4 and relevant clinicopathologic characteristics, including 5-year survival. In the present study, we did not find a significant relationship between MCM 7 and Smad 4 expression.

The MCM complex performs a pivotal role in the initiation and elongation of DNA replication. Recent studies have demonstrated increased MCM protein expression in esophageal squamous cell carcinoma, and the prognostic implications of this finding. As reported by Kato *et al*.,[17] the expression of MCM 2 was correlated with TNM stage. Huang *et al*.[18] assessed MCM 4 expression in 60 cases of esophageal cancer via reverse transcription polymerase chain reaction, and demonstrated significant differences in MCM 4 expression status between T3 stage and T1 stage groups. MCM 5 levels were also evaluated by Williams *et al*.[19] in gastric aspiration specimens using immunofluorometric measurements. They showed that the elevation of MCM 5 levels was highly predictive of esophageal cancer in gastric aspirates. To the best of our knowledge, the expression of MCM 7 in esophageal cancer has not been determined. The present study is the first report of MCM 7 expression in esophageal squamous cell carcinoma.

This study did not evaluate the utility of MCM 7 as a prognostic marker in esophageal squamous cell carcinoma. Our results differ to some degree from those of previous studies in which MCM 7 was identified as a reliable diagnostic and prognostic marker. Fujioka *et al*.[11] evaluated MCM 7 labeling indices in 100 cases of lung adenocarcinoma of diameter less than 3 cm (pT1 stage), and demonstrated that higher levels of MCM 7 expression were correlated with poor differentiation of tumors, non-bronchioloalveolar carcinomas, large tumor size, and poor prognosis. Li *et al*.[12] also demonstrated that MCM 7 expression was significantly correlated with poor histologic grade, old age, and poor survival in cases of endometrial carcinoma. Padmanabhan *et al*.[13] previously demonstrated that MCM 7 had a significantly higher proliferation index than Ki 67, and that it was associated with tumor stage and perineural invasion in prostatic intraepithelial neoplasia and invasive adenocarcinoma. Our study was conducted using a limited number of specimens, as compared with those of Fujioka *et al*.[11] and Li *et al*.,[12] and this

Table 2. Relationship between expression of Smad 4 and clinicopathological parameters

Parameters	n	Negative (loss of expression) (%) (n = 18)	Positive (expression) (%) (n = 49)	p-value[a]
T stage				0.934
I	16	4 (25.0)	12 (75.0)	
II	16	5 (31.2)	11 (68.8)	
III	29	7 (24.1)	22 (75.9)	
IV	6	2 (33.3)	4 (66.7)	
Node metastasis				0.790
Absent	39	10 (25.6)	29 (74.4)	
Present	28	8 (28.6)	20 (71.4)	
Distant metastasis				1.000
Absent	65	18 (27.7)	47 (72.3)	
Present	2	0 (0.0)	2 (100.0)	
Histologic grade				0.585
Well differentiation	22	6 (27.3)	16 (72.7)	
Moderate differentiation	32	7 (21.9)	25 (78.1)	
Poor differentiation	13	5 (38.5)	8 (61.5)	
Location				0.286
Upper third	2	1 (50.0)	1 (50.0)	
Mid third	53	15 (28.3)	38 (71.7)	
Lower third	12	2 (16.7)	10 (83.3)	
Neural invasion				0.375
Absent	60	15 (25.0)	45 (75.0)	
Present	7	3 (42.9)	4 (57.1)	
Vascular invasion				1.000
Absent	66	18 (27.3)	48 (72.7)	
Present	1	0 (0.0)	1 (100.0)	
Lymphatic invasion				0.584
Absent	48	12 (25.0)	36 (75.0)	
Present	19	6 (31.6)	13 (68.4)	

[a]Significance level of the chi-square test.

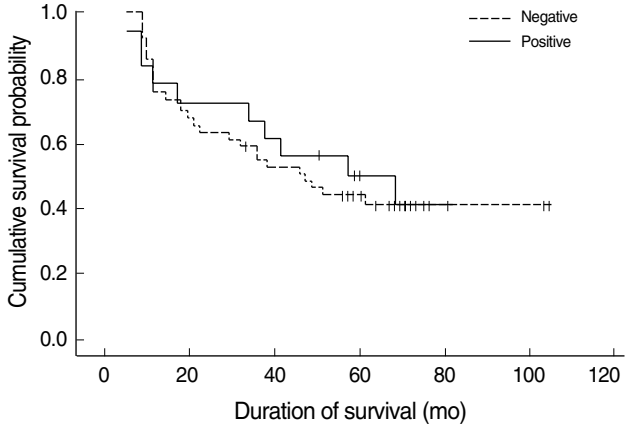

Fig. 4. Kaplan-Meier analysis of the overall survival of 67 patients with esophageal squamous cell carcinoma according to Smad 4 expression status. No significant difference is noted in overall survival between patients with tumors positive for Smad 4 and tumors negative for it (log-rank test, p = 0.798).

limitation contributed significantly to our failure to uncover evidence of a relationship between MCM 7 and survival.

Smad 4, a well-known tumor suppressor gene associated with pancreatic cancer, performs a crucial function in TGF-β superfamily signaling. Several previous studies have been conducted regarding the expression of Smad 4 and its clinicopathological significance in esophageal squamous cell carcinoma, but a role for Smad 4 in the development of esophageal cancer has not been established. Natsugoe *et al*.[29] reported that loss of Smad 4 expression is significantly associated with tumor depth and lymph node metastasis. Fukuchi *et al*.[20] discovered an inverse correlation between Smad 4 expression and invasion depth and pathologic stage. However, Osawa *et al*.[30] suggested that mutation of the *Smad 4* gene is a rare event in cases of esophageal cancer. This was based on the result of polymerase chain reaction-single strand conformation polymorphism analysis that did not show any genetic mutations related to Smad 4 or any other TGF-β/Smad pathway. An analysis of the data in the present study failed to detect any correlation between Smad 4 expression and relevant clinicopathological parameters. This result is consistent with the opinion of Osawa *et al*.[30] that Smad 4 has a limited role in

esophageal carcinoma.

There was no correlation between MCM 7 and Smad 4 expression in the present study. This suggests that another mechanism might activate MCM 7 in esophageal cancer. However, further studies are needed to verify this idea, since there is no report yet about the relationship of two proteins.

In this study, immunohistochemistry was the only method appropriate for the elucidation of MCM 7 and Smad 4 expression. The results of immunohistochemical staining were not necessarily consistent with the results of analyses of chromosome level, such as polymerase chain reaction. Fukuchi *et al*.[20] detected a relationship between Smad 4 expression and relevant prognostic parameters using immunohistochemistry, but did not detect significant Smad 4 mRNA expression via western and northern blot analyses. The results from this study on MCM 7 and Smad 4 expression in esophageal squamous cell carcinoma should be bolstered by complementary studies at the molecular level.

In summary, MCM 7 expression is correlated with depth of invasion in esophageal squamous cell carcinoma. Although no distinct correlation between MCM 7 and survival was noted in this study, our results indicate that MCM 7 is a marker for the invasiveness of esophageal squamous cell carcinoma. Furthermore, large-scale multivariate analysis studies are necessary to determine the prognostic implications of MCM 7 expression in cases of esophageal squamous cell carcinoma. Smad 4 appears to be of limited significance in esophageal squamous cell carcinoma.

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