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Evaluation of Tumor Malignancy in Esophageal Squamous Cell Carcinoma Using Different Characteristic Factors

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Abstract. *Background: We have been investigating various molecules correlated with the malignancy of esophageal squamous cell carcinoma and, in the present study, we examined the correlation of four of them (KAI1, FAK, EphA2, Ki-67 labeling index) with the prognosis of affected patients. Furthermore, the use of biopsy samples was studied to evaluate whether the grade of tumor malignancy can be determined before treatment in a clinical setting. Materials and Methods: Tissue specimens that had been surgically removed from 91 patients with thoracic esophageal cancer and 247 biopsy samples were examined. The malignancy index (MI) was defined in terms of the KAI1, FAK and EphA2 scores and the Ki-67 labeling index, and the reliability and utility of the correlation between MI and prognosis was evaluated. Results: The mean 5-year survival rate of patients with MI=0 was 100%, while that of patients with MI=1, 2 and 3 was 70%, 48% and 10%, respectively. Patients with MI=4 all died, with the exception of one who has been observed for 3 years. The rate of concordance between the biopsy samples and surgical specimens was 79.4% for KAI1, 88.2% for FAK and 73.5% for EphA2, and the rates of concordance for 1, 2, 3, 4, 5, 6, 7 and 8 biopsy samples were 66.7%, 64.1%, 74.5%, 90.7%, 91.7%, 83.3%, 100% and 100%, respectively. Conclusion: It may be feasible to evaluate the malignancy of tumor cells and to predict patient outcome by using multiple marker molecules. It is anticipated that such data will accelerate the development of "tailor-made" therapy.*

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Esophageal cancer is one of the most aggressive and lethal of malignancies. The mortality rates are very similar to the incidence rates (1) due to the relatively late stage of diagnosis and inefficiency of treatment; the survival rate at 5 years is reportedly <10% (2), although recent advances in surgical techniques and adjuvant therapy have improved the 5-year survival rate to about 40% (3). Some treatments (surgery, radiotherapy, chemotherapy, hyperthermia, *etc.*) have been combined to provide a multidisciplinary approach but, as the characteristics of cancer cells are very diverse, tumor responses to treatment vary considerably. Therefore, the survival of individual patients is influenced by a number of factors.

Patient survival invariably depends on the degree of malignancy of a cancer. Factors that reflect cancer malignancy, and thus prognosis, include tumor stage, treatment, sensitivity to treatment and the properties of the tumor cells themselves, such as growth speed, metastatic ability and invasion to other organs.

Recently, various molecular biological factors such as cyclin D1, E-cadherin epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) have been proposed as prognostic factors for esophageal cancer (4-8). However, none of these have been shown to be superior to pathological findings or to be reliable indicators of prognosis in a clinical setting.

We have previously studied some factors with potential utility for evaluating the malignancy of esophageal squamous cell carcinoma (ESCC) (9-11). KAI1/CD82, a tumor metastasis suppressor gene, is inversely correlated with the progression and invasion of several tumors (12-18). We have also demonstrated immunohistochemically that the expression of KAI1 protein is correlated with lymph node metastasis (9). EphA2 is a member of the Eph family of receptor tyrosine kinases (19). Recent studies have demonstrated EphA2 expression in several human tumors (20-23). A significant correlation was observed between

EphA2 expression and regional lymph node metastasis, the number of lymph node metastases and a poor degree of tumor differentiation. Focal adhesion kinase (p125FAK, hereafter referred to as FAK) is a tyrosine kinase localized to cellular focal adhesions, that is associated with a number of other proteins, such as the integrin adhesion receptor (24-27). Overexpression of FAK has been reported in a number of invasive human cancers (28-33). In our study (11), FAK protein was correlated with cell differentiation, depth of tumor invasion, the occurrence of regional lymph node metastasis and the number of lymph node metastases. Ki-67 is a nuclear antigen detected by the monoclonal antibody MIB-1 and its labeling index is considered a marker of tumor proliferation (34). Some studies have demonstrated a correlation between Ki-67 expression and the clinicopathological features and prognostic factors of ESCC (35-37).

As the above four factors are known to be correlated with the prognosis of patients with ESCC, we investigated the possibility of using them in combination to evaluate tumor malignancy. Furthermore, the use of biopsy samples was studied to evaluate whether the grade of tumor malignancy can be determined before treatment.

Materials and Methods

Patients and tissue samples. The tissue specimens used had been removed surgically from 91 patients with thoracic ESCC at Gunma University Hospital, Japan, between 1983 and 2001. Of these 91 cases, biopsy specimens were also available for 247 samples of 68 patients. Written informed consent to participate in the study was obtained from each patient before surgery, according to the ethical guidelines of our university. All patients underwent potentially curative surgery without preoperative therapy. There were 77 men and 14 women, aged 40-78 years (mean age: 61.0). The tumor stages were classified according to the 5th edition of the TNM classification of the International Union Against Cancer (UICC). Evaluation of tumor differentiation was based on the histological criteria in the guidelines of the Japanese Society for Esophageal Diseases (1999). The mean postoperative follow-up period was 71.8 months (range: 32.5-192.2).

The specimens were fixed in 10% formaldehyde solution and embedded in paraffin. Operative sections, that contained both a tumor-invasive portion and normal esophageal epithelium, and all biopsy specimens were examined for diagnosis of malignancy.

Antibodies. Antibodies were purchased from the following manufacturers: monoclonal antibody (Mab) specific for FAK (clone 4.47), Upstate Biotechnology Inc., Lake Placid, NY, USA; Mab specific for EphA2 (clone D7) (Upstate Biotechnology, Inc.); Mab specific for Ki-67 (MIB-1), Immunotech, Marseille, France; polyclonal antibody specific for KAI1 (c-17), Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Immunohistochemistry. Immunohistochemical staining was performed by the standard streptavidin-biotin (SAB) method. The details have been described previously (9-11).

Evaluation of immunostaining for four factors. For KAI1/CD82, when 10% or more of the carcinoma cells in a given specimen were positively-stained as well as the normal epithelium, the sample was classified as positive (+), and when <10% were stained, as negative (–) (9). For FAK, when 40% or more of the carcinoma cells in a given specimen were stained more intensely than the normal epithelium in the same section, the sample was classified as showing overexpression (FAK(+))(11). For EphA2, when 40% or more of the carcinoma cells in a given specimen were stained more intensely than the normal epithelium the sample was classified as positive (+) (10). The Ki-67 labeling index was calculated as the percentage of nuclear staining of cells at the invasive front of the tumor in three consecutive high-power fields (x400); each field corresponded to a total number of 300 to 1000 cells. At least 1000 cells were counted per sample. As the median Ki index is 38.7, a Ki index of 39 or more was classified as high (Ki-H), and one of <39 was classified as low (Ki-L).

Definition of malignancy index (MI). The aim was to evaluate esophageal malignancy in terms of KAI1, FAK and EphA2 expressions and the Ki index. In brief, we scored KAI1-negative cases as 1 point and positive cases as 0. Following the same rule, FAK-positive cases scored 1 point and negative cases 0, and EphA2-positive cases scored 1 point and negative cases 0. Ki-H cases scored 1 point and Ki-L cases 0. The malignancy index (MI) was defined as the total points score for the four factors.

Statistical analysis. Statistical analysis was performed using the unpaired two-group *t*-test for age, number of lymph node metastases and the Ki index. The χ^2 test was used for testing differences in gender, differentiation, location and TNM clinical staging. Survival curves were calculated by the Kaplan-Meier method and analysis was carried out by the log-rank test.

Results

Relationship between expressions of the four factors and clinicopathological features. The expressions of the four factors, KAI1, FAK, EphA2 and the Ki index, in ESCC was investigated by immunohistochemical analysis of formalin-fixed, paraffin-embedded specimens using each specific Mab. Among the 91 patients, KAI1/CD82 expression was positive in 48 (52.7%), EphA2 overexpression was detected in 45 (49.5%) and FAK overexpression was detected in 54 (59.3%). The relationship between the clinicopathological characteristics of patients with ESCC and the expressions of all four factors is summarized in Table I. A significant correlation was observed between KAI1 expression and depth of tumor invasion ($p=0.0071$), presence of regional lymph node metastasis ($p=0.0014$) and disease stage ($p=0.002$). A significant correlation was observed between EphA2 overexpression and the presence of regional lymph node metastasis ($p=0.0072$) and disease stage ($p=0.026$). A significant correlation was observed between FAK overexpression and cell differentiation ($p=0.0057$), depth of tumor invasion ($p=0.0023$), the presence of regional lymph node metastasis ($p=0.0097$) and disease stage ($p=0.012$). A

Table I. *The correlation between clinicopathological characteristics and expression of four factors.*

Parameters	Total	KAI1(+) n=48	KAI1(–) n=43	*p*-value	EphA2(–) n=46	EphA2(+) n=45	*p*-value	FAK(–) n=37	FAK(+) n=54	*p*-value	Ki (L) n=45	Ki (H) n=46	*p*-value
Age (mean±SD, yrs)	61.0±8.2	61.6±8.2	60.8±8.6	0.64	60.7±8.8	61.7±8.0	0.58	59.8±8.2	61.5±7.9	0.05	61.9±9.5	60.5±7.1	0.45
Gender													
Male	77	41	36		38	39		34	43		39	38	
Female	14	7	7	0.82	8	6	0.59	3	11	0.11	6	8	0.59
Differentiation													
Well	23	11	12		6	17		13	10		16	7	
Moderate	45	25	20		26	19		21	24		20	25	
Poor	23	12	11	0.83	13	10	0.35	3	20	0.0057	9	14	0.08
Location													
upper	12	5	7		4	8		6	6		5	7	
mid-thorax	56	34	22		32	24		21	35		31	25	
lower	23	9	14	0.16	9	14	0.17	10	13	0.69	9	14	0.36
TNM clinical classification													
T T1	35	26	9		13	22		20	15		21	14	
T2	13	6	7		5	8		8	5		5	8	
T3	37	15	22		23	14		9	28		16	21	
T4	6	1	5	0.0071	4	2	0.12	0	6	0.0023	3	3	0.43
N N0	37	27	10		25	12		21	16		24	13	
N1	54	21	33	0.0014	21	33	0.0072	16	38	0.0097	21	33	0.015
M M0	75	44	31		40	35		32	43		40	35	
M1	16	4	12	0.14	6	10	0.25	5	11	0.40	5	11	0.11
Stage													
I	24	20	4		17	7		15	9		15	9	
II	28	14	14		16	12		13	15		15	13	
III	23	10	13		7	16		4	19		10	13	
IV	16	4	12	0.002	6	10	0.026	5	11	0.012	5	11	0.23
Total	91	48	43		46	45		37	54		45	46	

SD: standard deviation

significant correlation was observed between the Ki index and the presence of regional lymph node metastasis ($p=0.015$).

The survival rates of patients with KAI1(–) cancer were significantly lower than those of patients with KAI1(+) cancer ($p=0.0023$; Figure 1A). The mean 5-year survival rate of patients with KAI1(+) cancer was 60%, whereas that of patients with KAI1(–) cancer was 24%. The survival rates of patients with EphA2(+) cancer were significantly lower than those of patients with EphA2(–) cancer ($p<0.0044$; Figure 1B). The mean 5-year survival rate of patients with EphA2(–) cancer was 60%, whereas that of patients with EphA2(+) cancer was 25%. The survival rates of patients with FAK(+) cancer were significantly lower than those of patients with FAK(–) cancer ($p<0.0001$; Figure 1C). The mean 5-year survival rate of patients with FAK(–) cancer was 69%, whereas that of patients with FAK(+) cancer was 23%. The survival rates of patients with Ki-H cancer were significantly lower than those of patients with Ki-L cancer ($p=0.0264$; Figure 1D). The mean 5-year survival rate of patients with Ki-L cancer was 58%, whereas that of patients with Ki-H cancer was 33%.

With a view to evaluating tumor malignancy, the degree of protein expression of the four factors was scored, and the total points score for the four factors was defined as the malignancy index (MI). MI was 0 in 6 cases, 1 in 20 cases, 2 in 38 cases, 3 in 19 cases and 4 in 8 cases. The mean 5-year survival rate of MI=0 patients was 100%, and that of patients with MI=1, 2 and 3 was 70%, 48% and 10%, respectively (Figure 2). Patients with MI=4 all died, with the exception of one who has been observed for 3 years.

Rate of concordance between biopsy samples and surgical specimens. Of the 91 cases, biopsy specimens were evaluable for 68, and the number of biopsy specimens per case ranged from 1 to 8 (mean 3.6). The rate of concordance between the biopsy samples and surgical specimens for each of the factors was KAI1 79.4%, FAK 88.2% and EphA2 73.5%.

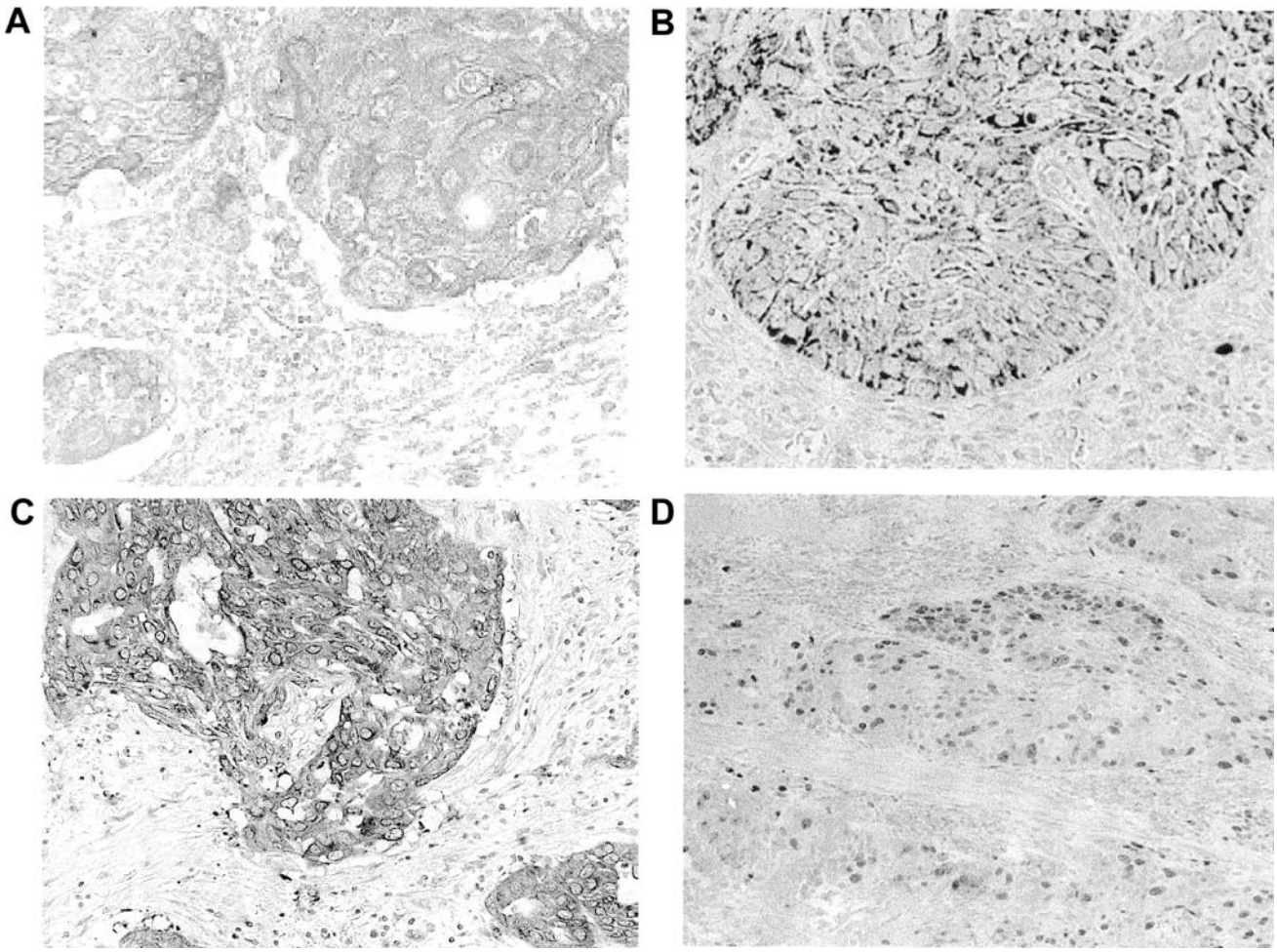

Figure 1. *Representative photomicrographs of tissue sections immunostained for four molecules. (A) KAI-1 was detected in the cell membrane and cytoplasm of the normal esophageal epithelium and cancer tissue. (B) EphA2 protein overexpression was detected in invasive cancer fronts, particularly in cells located in the peripheral layers of cancer cell nests. (C) Small scattered clusters of cancer cells expressed FAK protein abundantly. (D) Ki-67 was detected in the cell nucleas of cancer tissue.*

The rate of concordance increased with the number of available biopsy specimens (Figure 3). The rates of concordance for 1, 2 and 3 biopsy samples were 66.7%, 64.1% and 74.5%, respectively, and those for 4, 5, 6, 7 and 8 samples were 90.7%, 91.7%, 83.3%, 100% and 100%, respectively.

Discussion

Our immunohistochemical results suggested that a combination of predictive factors accurately reflected the survival of patients. MI may, therefore, be a good prognostic indicator in patients with esophageal cancer. Various factors have already been proposed to have prognostic value. However, the use of different factors in combination may provide more accurate information on prognosis, metastasis and/or recurrence.

Individual factors have been thought to play different roles in normal cells. The KAI1 gene product is identical to CD82, a surface glycoprotein of leukocytes, which comprises 267 amino acids. It belongs to a structurally distinct family of membrane glycoproteins (the transmembrane-4 superfamily). Prostate carcinomas with low levels of KAI1/CD82 gene expression show more aggressive biological characteristics than those with high levels (12). It has been reported that pancreatic (13), bladder (14), breast (15), non-small cell lung (16), gastric (17) and esophageal (18) carcinomas have similar characteristics. EphA2 (M_r 130,000) is a member of the Eph family of receptor tyrosine kinases, which interact with

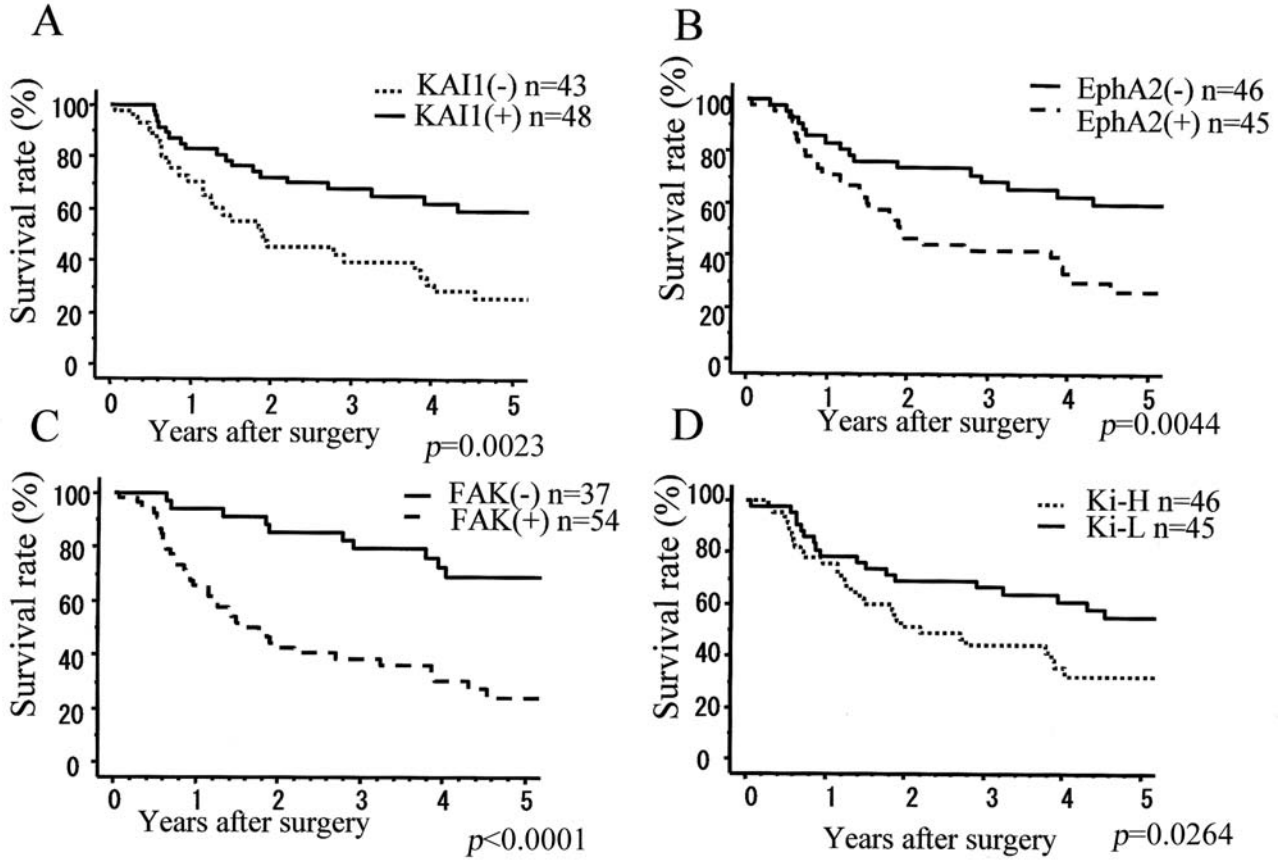

Figure 2. *Relationship between overall postoperative survival and expression of four factors. (A) KAI1-expression (+) patients had a significantly more favorable prognosis than those without KAI1 expression (5-year survival rates: KAI1(–), 24%; KAI1(+), 60%; p=0.0023). (B) EphA2-overexpression (–) patients had a significantly more favorable prognosis than those with EphA2 overexpression (+) (5-year survival rates: EphA2 overexpression (+), 25%; EphA2 overexpression (–), 60%; p=0.0044). (C) FAK-overexpression (–) patients had a significantly more favorable prognosis than those with FAK overexpression (+) (5-year survival rates: FAK overexpression (+), 23%; FAK overexpression (–), 69%; p<0.0001). (D) Ki-index low patients had a significantly more favorable prognosis than those with Ki-index high (5-year survival rates: Ki-H, 33%; Ki-L, 58%; p=0.0264).*

cell-bound ligands known as ephrins (19). The function of Eph kinase has been studied in detail in normal cells and is suggested to regulate cell proliferation, differentiation and migration. Therefore, it has also been suspected that Eph kinase might be associated with the degree of malignancy of cancer. Recent studies have demonstrated EphA2 expression in human melanoma, (20) colon cancer, (21) prostate cancer, (22) and mammary cancer (23). A cell line study has also indicated that EphA2 is a powerful oncoprotein in breast cancer and that its overexpression causes malignant transformation (23). In the present study, a relationship was observed between EphA2 overexpression and both lymph node metastasis and unfavorable prognosis. FAK is a tyrosine kinase localized to cellular focal adhesions, that is associated with a number of other proteins. FAK is involved in the integrin-signaling pathway (24-26), cellular motility (38, 39) and apoptosis (40-42). Overexpression of FAK has been reported in a number of invasive human cancers (28-33) and, in some cases, there has been a suspected relationship between FAK expression and metastatic ability. The Ki-67 labeling index is considered to be a marker of tumoral proliferation, which in turn is thought to be correlated with tumor malignancy. Some reports have shown a correlation between the expression of Ki-67 and clinicopathological features and prognostic factors in ESCC (35-37). Because the degree of malignancy of cancer cells involves many factors, the properties of the above marker molecules were investigated in more detail. Some studies have demonstrated that prognosis and/or tumor metastasis are determined by a number of molecules (43, 44). Therefore, in the present study, the possibility of using a combination of different marker molecules to assess the degree of tumor malignancy by molecular biological techniques was evaluated.

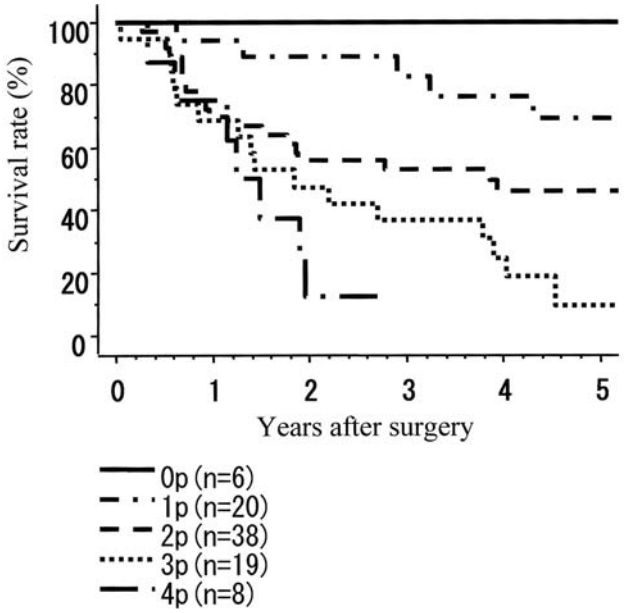

Figure 3. *Relationship between overall postoperative survival and MI. The mean 5-year survival rate of patients with MI=0 was 100%, that of patients with MI=1, 2 and 3 was 70, 48 and 10%, respectively. All patients with MI=4 died, with the exception of one who has been observed for 3 years.*

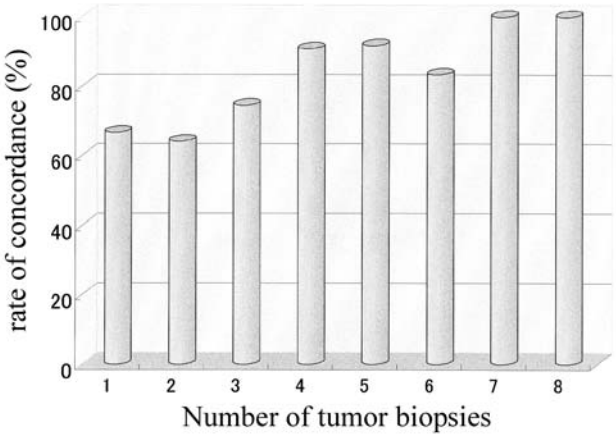

Figure 4. *Rate of concordance between biopsy samples and operation specimens. Relationship between numbers of biopsy samples and rate of concordance. Rates of concordance of 1, 2, 3, 4, 5, 6, 7 and 8 were 66.7, 64.1, 74.5, 90.7, 91.7, 83.3, 100 and 100%, respectively.*

Pathological (TNM) staging has been demonstrated to be one of the most accurate prognostic tools available, but has a disadvantage in that no information can be obtained without surgery. Ideally, it would be desirable to obtain such information before treatment, because it might offer an opportunity for less invasive approaches such as neoadjuvant therapy. Therefore, in our present series, biopsy samples that had been obtained before treatment were also studied.

The rate of concordance depends on the expression pattern of each factor and the number of biopsy samples obtained. The rate of concordance of the four factors examined was 70-80%, and this rate appeared to increase with the number of available biopsy samples.

As the four molecules investigated each play different roles in normal cells, we considered that they might have potential for evaluating the malignancy of ESCC from different viewpoints (*i.e.* growth, motility, invasiveness, *etc.*). Our findings suggest that these factors may have potential for evaluating both the malignancy of tumor cells and patient prognosis. It is anticipated that the presented data will be useful for the development of "tailor-made" therapy for individual patients.

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