

Co-expression of aFGF and FGFR-1 is predictive of a poor prognosis in patients with esophageal squamous cell carcinoma

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Abstract. Overexpression of aFGF, bFGF and FGFR-1 has been reported in various cancers, and it has been suggested that it may be a poor prognostic factor in cases with solid tumors. Therefore, we attempted to determine whether overexpression of aFGF, bFGF and FGFR-1 might also be a poor prognostic factor in patients with esophageal squamous cell carcinoma, and examined the expression of aFGF, bFGF and FGFR-1 in esophageal cancer tissue specimens to clarify their clinical significance. Seventy-nine patients with squamous cell carcinoma of the esophagus who underwent resection at the Department of Surgery, Keio University Hospital, were enrolled as the subjects of this study. None of the patients had received any previous treatment. Formalin-fixed and paraffin-embedded sections of esophageal cancer tissue were stained by immunohistochemical methods and examined for expression of the angiogenetic factors and their receptors, and also to determine the microvascular density (MVD). We examined the correlations between the expression of aFGF, bFGF and FGFR-1, and the MVD, clinicopathological background factors and survival of the patients by conducting statistical analyses of the data. The results revealed that positive aFGF expression was associated with a larger tumor area ($p=0.009$), and co-expression of both aFGF and FGFR-1 was associated with a larger tumor area ($p=0.01$) and poorer prognosis ($p=0.04$). There were positive correlations between the expression of aFGF and FGFR-1 ($p<0.0001$), and between those of bFGF and FGFR-1 ($p=0.04$). aFGF may promote proliferation of esophageal cancer cells in an angiogenesis-independent and autocrine manner, and may contribute to rapid growth of esophageal cancer on recurrence after esophageal resection.

Introduction

The current staging of esophageal cancer is based on the UICC (International Union Against Cancer) TNM classification, and the surgical pathological findings have proven to be valuable (1). The pN factor is presently considered to be the most useful predictor of outcome in cases of squamous cell carcinoma of the esophagus, and 5-year survival rates of 61-80% and 34-45% have been reported for the pN0 status and pN1 status, respectively (2,3). Although there is clear evidence that patients with early-stage esophageal cancer do relatively well when treated by surgical resection alone, we have sometimes encountered even earlier-stage cancer patients who have developed recurrent disease and died after a curative resection; recurrence rates of 15% in cases with pN0 esophageal cancer and 14% in cases with pStage I disease have been reported (4). These observations suggest that the TNM classification alone may not be sufficient for accurate prediction of the prognosis in esophageal cancer patients, and new indicators of the biological malignant potential of squamous cell carcinoma of the esophagus must be explored (5).

It has been reported that cancer development depends on a variety of physiological processes, such as carcinogenesis at the cell oncogene level, proliferation, and tumor growth and progression. Tumor angiogenesis, as the means of supply of oxygen and nutrients, is necessary for tumor growth, and also for tumor progression, because it increases the opportunity for the tumor cells to enter the circulation (6). It has been demonstrated that the greater the number of tumor vessels, the greater the opportunity for tumor cells to enter the circulation (7), and that newly formed capillaries are more easily penetrated than mature vessels (8). Thus, the relationship between tumor angiogenesis and tumor growth has already been demonstrated, and a new advanced anti-tumor angiogenesis strategy has been attempted for cancer therapy (9-10).

As angiogenetic factors are secreted from cancer cells, the expression levels of VEGF, aFGF (acidic FGF; FGF-1) and bFGF (basic FGF; FGF-2) in cancers of various organs were investigated, and their correlations with the MVD (microvascular density) were examined. The expression levels of the VEGF, aFGF and bFGF receptors in various cancers were also reported. The FGF family consists of 10 members (FGF-1-10) and 4 FGF-homologous factors, and comprises a group of proteins with related functions (11-13). These polypeptides have been shown to act as mitogens for

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numerous cell types derived from the mesoderm and neurectoderm, and also for cancer cells (14-16). FGFs are well known as not only endothelial growth factors, but also as angiogenesis inducing factors. They act through high-affinity binding sites that mediate biological activity via a group of tyrosine kinase membrane receptors from the FGFR family. The FGFRs are encoded by at least 4 kinds of genes and possess immunoglobulin-like extracellular domains (17).

Overexpression of aFGF, bFGF and FGFR-1 in various cancers has been reported, and it has been suggested that it may be a poor prognostic factor in cases with solid tumors, including breast cancer (18-19), glioblastoma (20), hepatocellular carcinoma (21,22), and pancreatic carcinoma (23). Furthermore, numerous studies have suggested that proliferation of cancer cells might be promoted in an autocrine manner, based on the observation of a correlation between aFGF and FGFR-1 expression in breast cancer (19), and between bFGF and FGFR-1 in hepatocellular carcinoma (21).

Based on these previous observations, it was expected that overexpression of aFGF, bFGF and FGFR-1 might represent a prognostic factor in patients with esophageal squamous cell carcinoma, and we examined the expression of aFGF, bFGF and FGFR-1 in resected specimens of esophageal squamous cell carcinoma and their clinical significance, and also clarified the correlation between the expression levels of aFGF and FGFR-1, and those of bFGF and FGFR-1 in esophageal squamous cell carcinoma. Our results suggested that the cancer cell proliferation in esophageal squamous cell carcinoma might be promoted in an autocrine manner.

Materials and methods

Clinical materials. Seventy-nine patients with esophageal squamous cell carcinoma who had no history of previous treatment and underwent esophagectomy at Keio University Hospital (Tokyo, Japan) between January 1990 and December 1993 were enrolled for the study. Of the 79 patients, 68 were male and the remaining 11 were female. The patients ranged in age from 36 to 83 years, and their average age was 61.5 years. The pathological examinations were performed according to the Guidelines for Clinical and Pathological Studies on Carcinoma of the Esophagus of the Japanese Society for Esophageal Diseases (24). Based on the above, category pT1b (n=22) corresponds to tumors that have invaded the submucosa, category pT2 (n=13) corresponds to tumors that have invaded the muscularis propria, category pT3 (n=44) corresponds to tumors that have invaded the adventitia, category pN0 (n=28) corresponds to tumors with no regional lymph node metastasis, and category pN1 (n=51) corresponds to tumors with regional lymph node metastasis. Patients were followed-up at the outpatient clinic, and diagnostic examinations consisting of chest X-ray, chest and abdominal computed tomography, and abdominal ultrasonography were performed every 6 months to detect recurrences. The maximum patient follow-up period was 147 months, and the mean observation period was 46 months.

Immunohistochemical staining. Ten- μ m sections were made from 10%-formalin-fixed, paraffin-embedded blocks and

mounted on slides. The blocks were selected from the most invasive area of the carcinoma according to the pathology report. Sections were deparaffinized and rehydrated and then digested in 1% trypsin in calcium chloride for 30 min at 37°C. The tissue sections were covered with 3% H₂O₂ in methanol for 5 min and incubated for 60 min in bovine serum albumin (BSA) to suppress nonspecific IgG binding. The sections were then incubated with a 1:100 dilution of the primary antibody (aFGF, bFGF and FGFR-1), and a 1:200 dilution of the antibody for von Willebrand factor (primary antibody used for measurement of the MVD) in phosphate-buffered saline (PBS) at 4°C for 24 h. A labeling streptavidin biotin (LSAB) kit (Dako, Glostrup, Denmark) was used for immunohistochemical staining. Diamino benzidine tetrahydrochloride (DAB) was used as the chromogen, and the sections were counterstained with Meyer's hematoxylin.

Antibodies. The primary antibodies were monoclonal anti-bovine aFGF antibody (Upstate Biotechnology Inc., USA), monoclonal anti-bovine bFGF antibody (Upstate Biotechnology Inc.), monoclonal anti-human FGFR-1 antibody (Santa Cruz Inc., USA), and polyclonal antihuman von Willebrand factor antibody (Dako) for the MVD analysis.

Staining analysis. The staining areas with the monoclonal antibodies for FGF, bFGF and FGFR-1 tended to be either <30% or >80%. If the cytoplasm of the cancer cells were stained >30% (aFGF and FGFR-1) or >80% (bFGF), we judged the staining as 'positive' (5). We defined MVD as the count of microvasculars per square-millimeter in the 'vascular hot spot' in the deepest cancer sections. Cases with a calculated density of more than 60/mm² were considered to have a high MVD (5).

Statistical analysis. The χ^2 test was used to evaluate the differences in the background factors among the patient groups. The cumulative survival rates for the patient groups were calculated by the Kaplan-Meier method, and compared using the log rank test. The influence of each variable on the patients' survival was assessed by the Cox proportional-hazards regression model. Statistical significance was defined as $p < 0.05$. We used StatView for Windows, Version 5.0 (SAS Institute Inc.), for all the analyses.

Results

According to the results of immunohistochemical staining, aFGF and bFGF were expressed mainly in the cytoplasm and perinuclear areas of the cancer cells and the stromal fibroblasts, and FGFR-1 was expressed mainly in the cytoplasm of the cancer cells and stromal fibroblasts (Fig. 1). The expression of all of the three molecules, aFGF, bFGF and FGFR-1, was relatively weak in the endothelial cells.

Forty-seven (59%) of the 79 cases were evaluated as aFGF-positive, 43 (54%) as bFGF-positive, and 47 (59%) as FGFR-1-positive.

Thirty-five (44%) as both aFGF- and FGFR-1-positive, and 22 (28%) as both aFGF- and FGFR-1-negative. Twenty-six (33%) were evaluated as both bFGF- and FGFR-1-positive, and 18 (23%) as both- bFGF and FGFR-1-negative.

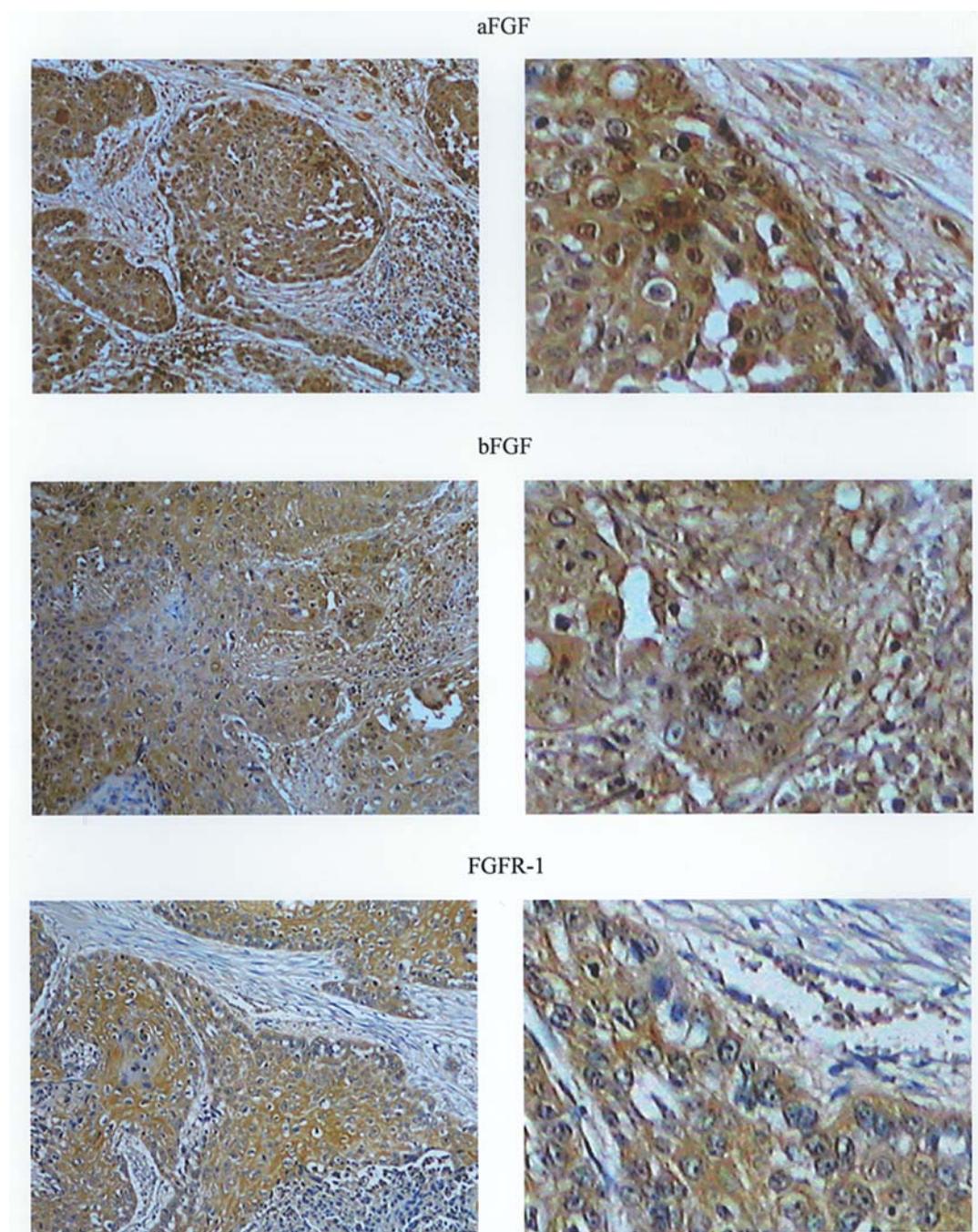


Figure 1. Immunohistochemical staining. The expression of aFGF and bFGF was identified mainly in both the cytoplasm and perinuclear areas of the cancer cells, and stromal fibroblasts. The expression of FGFR-1 was identified mainly in the cytoplasm of the cancer cells and stromal fibroblasts.

The patients were divided into groups, as follows, based on the above findings: an aFGF-positive group and aFGF-negative group; a bFGF-positive group and bFGF-negative group; a FGFR-1-positive group and FGFR-1-negative group; a aFGF- and FGFR-1-positive group and an aFGF- and FGFR-1-negative group; a bFGF- and FGFR-1-positive group and a bFGF- and FGFR-1-negative group. The following clinicopathological background factors were compared in each of the paired groups: age, sex, location of the tumor, operative approach (RTTE or THE), postoperative therapy (chemotherapy or radiation), tumor depth, differentiation, infiltration, lymphatic invasion, venous invasion, intraepidermal expansion, intramural metastasis, lymph node

metastasis, pathological stage, MVD, tumor area [calculated as the longest axis (mm) multiplied by the shortest axis (mm) on the tumor surface] and number of lymph node metastases (Table I). There were no significant differences in the clinical background factors between any of the two groups. The aFGF-positive group had a larger tumor area than the aFGF-negative group ($p=0.009$). The aFGF- and FGFR-1-positive group also had a larger tumor area than the aFGF- and FGFR-1-negative group ($p=0.01$).

The correlations between the expression of aFGF and that of FGFR-1, and between the expression of bFGF and that of FGFR-1 were analyzed (Table II). There were positive correlations between the expression of aFGF and that of

Table I. Clinicopathological backgrounds.

Factors	aFGF positive (n=47/79)	p-value	bFGF positive (n=43/79)	p-value	FGFR-1 positive (n=47/79)	p-value	aFGF+FGFR-1 positive (n=35/57)	p-value	bFGF+FGFR-1 positive (n=26/44)	p-value
Age										
-49	8/11	N.S.	7/11	N.S.	9/11	N.S.	6/7	N.S.	6/8	N.S.
50-59	13/24		17/24		12/24		8/15		8/12	
60-69	11/21		10/21		11/21		8/16		4/8	
70-	15/23		9/23		15/23		13/19		8/16	
Sex										
Male	40/68	N.S.	37/68	N.S.	43/68	N.S.	31/49	N.S.	23/37	N.S.
Female	7/11		6/11		4/11		4/8		3/7	
Location										
Upper	8/11	N.S.	8/11	N.S.	9/11	N.S.	7/8	N.S.	7/8	N.S.
Middle	28/46		26/46		26/46		21/35		15/26	
Lower	11/22		9/22		12/22		7/14		4/10	
Operation										
RTTE	36/60	N.S.	35/60	N.S.	37/60	N.S.	26/41	N.S.	21/32	N.S.
THE	11/19		8/19		10/19		9/16		5/12	
Adjuvant C/RT										
(-)	36/57	N.S.	31/57	N.S.	34/57	N.S.	27/43	N.S.	19/33	N.S.
(+)	11/22		12/22		13/22		8/14		7/11	
Tumor depth										
pT1b	11/22	N.S.	11/22	N.S.	11/22	N.S.	9/18	N.S.	7/15	N.S.
pT2	6/13		7/13		8/13		5/9		4/7	
pT3	30/44		25/44		28/44		21/30		15/22	
Differentiation										
Well	8/14	N.S.	7/14	N.S.	8/14	N.S.	7/12	N.S.	5/9	N.S.
Moderate	37/61		34/61		37/61		28/44		21/33	
Poor	2/4		2/4		2/4		0/1		0/2	
Infiltration										
α	9/17	N.S.	7/17	N.S.	7/17	N.S.	6/14	N.S.	4/12	N.S.
β	31/51		29/51		32/51		23/35		19/29	
γ	7/11		7/11		8/11		6/8		3/3	
Lymphatic invasion										
(-)	7/14	N.S.	4/14	N.S.	6/14	N.S.	5/12	N.S.	3/10	N.S.
(+)	40/65		39/65		41/65		30/45		23/34	
Venous invasion										
(-)	34/59	N.S.	36/59	N.S.	36/59	N.S.	26/43	N.S.	22/34	N.S.
(+)	13/20		7/20		11/20		9/14		4/10	
Intraepidermal expansion										
(-)	25/40	N.S.	25/40	N.S.	27/40	N.S.	19/29	N.S.	14/20	N.S.
(+)	22/39		18/39		20/39		16/28		12/24	
Intramural metastasis										
(-)	40/70	N.S.	37/70	N.S.	42/70	N.S.	29/48	N.S.	22/38	N.S.
(+)	7/9		6/9		5/9		6/9		4/6	
Lymph node metastasis										
(-)	14/28	N.S.	12/28	N.S.	15/28	N.S.	12/24	N.S.	7/16	N.S.
(+)	33/51		31/51		32/51		23/33		19/28	

Factors	aFGF		bFGF		FGFR-1		aFGF+FGFR-1		bFGF+FGFR-1	
	positive (n=47/79)	p-value	positive (n=43/79)	p-value	positive (n=47/79)	p-value	positive (n=35/57)	p-value	positive (n=26/44)	p-value
Pathological stage										
I	6/14	N.S.	6/14	N.S.	6/14	N.S.	5/12	N.S.	3/9	N.S.
IIA	7/14		6/14		9/14		7/12		4/7	
IIB	12/18		13/18		12/18		9/13		7/10	
III	13/19		14/19		14/19		10/12		9/11	
IV	9/14		8/14		6/14		4/8		3/7	
MVD										
Low	30/54	N.S.	27/53	N.S.	34/54	N.S.	33/53	N.S.	40/66	N.S.
High	15/25		14/26		15/25		16/26		10/13	
Tumor area ^a		0.009		N.S.		N.S.		0.01		N.S.
Number of lymph node metastasis ^a		N.S.								

^aMann-Whitney's U tests were used. In clinical backgrounds, there were no significant differences between each of the two groups. The aFGF positive group had a larger tumor area than the aFGF negative group ($p=0.009$). The aFGF and FGFR-1 positive group had a larger tumor area than the negative group ($p=0.01$).

Table II. Correlations between ligands and receptors.

Ligand/ receptor	aFGF			bFGF		
	+	-	Total	+	-	Total
FGFR-1						
+	39	8	47	30	17	47
-	8	24	32	13	19	32
Total	47	32	79	43	36	79
p-value	<0.0001			0.04		

There were positive correlations between expression of aFGF and FGFR-1 ($p<0.0001$), and between bFGF and FGFR-1 ($p=0.04$).

FGFR-1 ($p<0.0001$), and between the expression of bFGF and that of FGFR-1 ($p=0.04$).

Distribution of the expression of aFGF and that of FGFR-1, and the expression of bFGF and that of FGFR-1 were analyzed (Table III). aFGF and FGFR-1, in particular, showed both strong expression and weak expression ($p<0.0001$). There was a positive correlation between the expression of bFGF and that of FGFR-1 ($p=0.02$).

Cumulative Kaplan-Meier survival curves for patients with aFGF-positive and aFGF-negative tumors, FGFR-1-positive and FGFR-1-negative tumors, and aFGF- and FGFR-1-positive and aFGF- and FGFR-1-negative tumors were calculated (Fig. 2). The survival rate in the aFGF- and FGFR-1-positive group was significantly shorter than that in the aFGF- and FGFR-1-negative group ($p=0.04$), and the rate in the aFGF-positive group tended to be shorter than in the

aFGF-negative group ($p=0.06$). The survival rates were not significantly different between the bFGF-positive group and the bFGF-negative group ($p=0.60$), between the FGFR-1-positive group and the FGFR-1-negative group ($p=0.11$), and between the bFGF- and FGFR-1-positive group and the bFGF- and FGFR-1-negative group ($p=0.43$).

The prognostic value of co-expression of aFGF and FGFR-1 and the MVD in the patients with esophageal squamous cell carcinoma was compared with that of the other clinicopathological predictive factors, such as tumor depth, tumor area and lymph node metastasis. The effects of variables associated with the prognosis were assessed by multivariate analysis using Cox's proportional hazards model, and the results of the multivariate analysis showed that the hazards ratio of the co-expression of both aFGF and FGFR-1 was the second highest, second only to lymph node metastasis, and that for the MVD was the fourth highest (Table IV).

Discussion

In this study, we clarified that there is a strong positive correlation between the expression of aFGF and FGFR-1 in esophageal squamous cell carcinoma. Co-expression of aFGF and FGFR-1 may be associated with the regulation of cell proliferation in an autocrine manner, and thereby contribute to poor prognosis. Consistent with this notion, our analysis results also indicated that co-expression of aFGF and FGFR-1 was associated with a poor prognosis in patients with esophageal squamous cell carcinoma. Numerous studies have reported correlations between prognosis and the expression status of aFGF and bFGF in a variety of cancer tissues: bFGF expression was associated with a shorter survival in cases of pancreatic ductal carcinoma (25), and

Table III. Distribution of expression of aFGF and FGFR-1 and expression of bFGF and FGFR-1.

	aFGF stained				bFGF stained			
	<30%	≥30%, <80%	≥80%	Total	<30%	≥30%, <80%	≥80%	Total
FGFR-1 stained								
<30%	24	3	5	32	10	9	13	32
≥30%, <80%	4	6	4	14	2	6	6	14
≥80%	4	6	23	33	4	5	24	33
Total	32	15	32	79	16	20	43	79
p-value	<0.0001						0.02	

aFGF and FGFR-1 distributed to both strong expression or both weak expression.

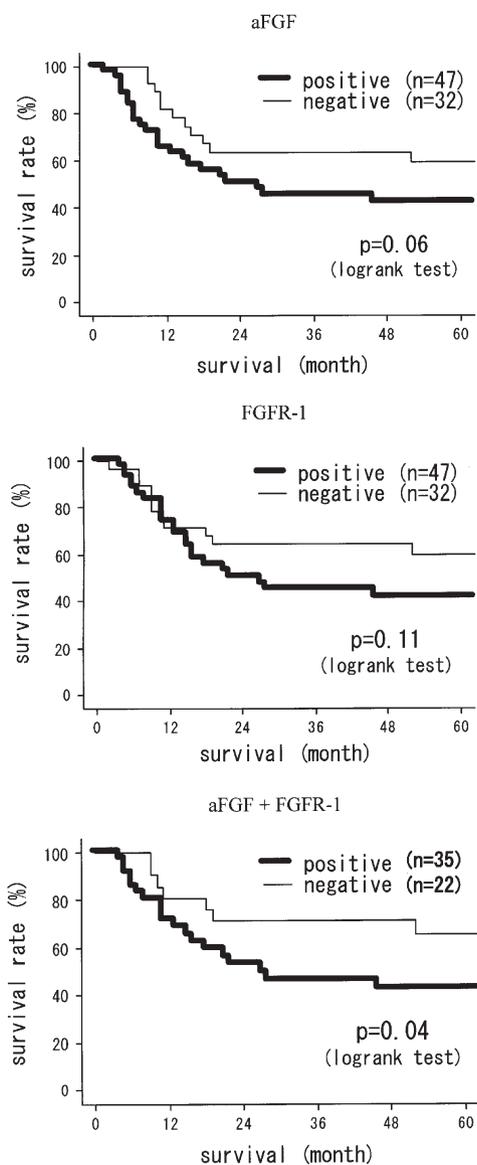


Figure 2. Kaplan-Meier survival curves. Survival was not significantly different between aFGF-positive and -negative groups ($p=0.06$), and between FGFR-1-positive and -negative groups ($p=0.11$). However, survival was significantly poorer in patients with both aFGF- and FGFR-1-positive tumors than both aFGF- and FGFR-1-negative tumors ($p=0.04$).

Table IV. Multivariate analysis using Cox's proportional hazards model.

Factors	Hazards ratio	p-value
pN (0 or 1)	4.87	0.02
aFGF+FGFR-1 (positive or negative)	2.49	0.14
pT (1,2 or 3)	2.19	0.15
MVD (count/mm ²)	1.01	0.40
Tumor area (mm ²)	1.00	0.64

The results showed that co-expression of aFGF and FGFR-1 had the second highest hazard ratio after the lymph node metastasis factor, and MVD had the fourth highest hazard ratio.

longer survival in cases of primary breast cancer (26), and aFGF expression was associated with an equivalent survival in cases of hepatocellular carcinoma (22).

Univariate analysis in our study showed that while the expression of aFGF, bFGF or FGFR-1 alone did not contribute to poor prognosis, co-expression of aFGF and FGFR-1 was associated with poor prognosis. Multivariate analysis showed that the hazard ratio for co-expression of aFGF and FGFR-1 was the second highest, second only to that for lymph node metastasis. While the pN factor has been reported to be the most useful predictor of the outcome in cases of esophageal squamous cell carcinoma. (2-3), our study showed that co-expression of aFGF and FGFR-1 was the second important predictor of a poor prognosis in cases of esophageal squamous cell carcinoma, independent of the pT factor, pN factor and the MVD. These findings suggest that co-expression of aFGF and FGFR-1 may promote cancer cell proliferation in esophageal cancer in an angiogenesis-independent autocrine manner.

On the other hand, while a positive correlation was noted between the expression of bFGF and FGFR-1 in our cases of esophageal squamous cell carcinoma, co-expression of bFGF and FGFR-1 was not associated with a poor prognosis.



SPANDIDOS d be explained by the poorer affinity of bFGF, as to that of aFGF, for FGFR-1 (27); other mechanisms inhibiting tumor growth under these circumstances may also be operative. Further investigation is required to characterize these associations.

Some studies which examined the expression of aFGF, bFGF and FGFR-1 by immunohistochemical staining and revealed correlations between ligand and receptor expression, suggest the existence of an autocrine regulatory mechanism for cancer proliferation (19,21). The relationship between the production of angiogenic factor and tumor growth represents one of the most important aspects in the study of carcinogenesis (28). aFGF and bFGF are unique as, in addition to being angiogenic factors, they are also epithelial, mesodermal and neuroectodermal mitogens (24). A number of studies have shown that alterations in the expression of FGF may contribute to growth deregulation in neoplastic cells (29-32).

Numerous *in vitro* studies have suggested the existence of an autocrine regulatory mechanism for cancer growth: the role of aFGF and bFGF in tumor development is supported by observations that cells transfected with the aFGF or bFGF gene show increased autocrinally promoted growth in monolayer cultures and soft agar (33,34). Studies employing neutralizing antibodies and anti-sense oligonucleotides which can attenuate FGF activity indicate that endogenous aFGF and bFGF may promote neoplastic cell growth in an autocrine manner (35,36). HSY human salivary-gland adenocarcinoma cells produce and utilize endogenous aFGF and bFGF for autocrine growth via an extracellular mode of action (37). However, to the best of our knowledge, there are no published studies on the expression of aFGF, bFGF and FGFR-1 in esophageal squamous cell carcinoma cell lines. To validate our hypothesis, therefore, we considered it necessary to examine the correlations between the expression of aFGF and FGFR-1 and cancer cell proliferation.

To explain why the co-expression of aFGF and FGFR-1 in our cases of esophageal carcinoma showed no correlations with the pT or pN status but was associated with a poor prognosis, we considered that the pathological stage (pT, pN) of resected esophageal squamous cell carcinoma did not reflect the rate of cancer progression. After resection, survival directly depends on the rate of development proliferation of the residual cancer cells. If rapid proliferation of the residual cancer cells after resection in cases of esophageal cancer was promoted in an autocrine manner, it could reasonably be surmised that survival would depend not on the pathological stage, but on the expression status of aFGF and FGFR-1.s

Acknowledgements

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