Expression of receptor tyrosine kinases in esophageal carcinosarcoma

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Abstract. Esophageal carcinosarcoma (ECS) is a rare malignant neoplasm associated with a poor patient prognosis. It is characterized by the presence of both malignant epithelial and mesenchymal components. Molecular-targeted therapy of several receptor tyrosine kinases (RTKs) has been reported to be effective in the treatment of various malignant tumors, including carcinosarcoma of several organs. This study aimed to assess the therapeutic potential of targeting RTKs in ECS. Overexpression of RTKs was assessed in 21 ECS cases by immunohistochemistry (IHC). Positively stained cases were further examined for RTK gene mutations and amplifications by direct sequencing analysis and fluorescence *in situ* hybridization. In epithelial components, KIT, platelet-derived growth factor receptor (PDGFR)A, PDGFRB, MET, epidermal growth factor receptor (EGFR) and HER-2 were overexpressed in 1 (4.8%), 1 (4.8%), 0 (0%), 11 (52.4%), 13 (61.9%) and 2 (9.5%) cases, respectively. In the mesenchymal components the corresponding numbers of cases were 2 (9.5%), 2 (9.5%), 0 (0%), 12 (57.1%), 11 (52.4%) and 0 (0%). No mutations in the *c-kit*, *PDGFRA* and *c-met* genes were found. Among 19 EGFR-positive tumors, 2 had EGFR missense mutations (T790A, exon 20) only in the mesenchymal component. Gene amplification or high polysomy of *c-kit*, *PDGFRA*, *c-met* and *EGFR* was observed in 1 (33.3%), 0 (0%), 3 (18.8%) and 10 (52.6%) cases, respectively. In conclusion, various RTKs, particularly MET and EGFR were overexpressed in ECSs suggesting that molecular-targeted therapies directed to MET, EGFR or other RTKs may be effective in inhibiting the growth or progression of the epithelial and/or mesenchymal component of ECS.

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Introduction

Carcinosarcoma, formerly called spindle-cell, pseudosarcomatous or sarcomatoid carcinoma (1), is a rare biphasic tumor characterized by a combination of malignant epithelial and mesenchymal cell proliferations. These tumors occur in various organs, including the upper aerodigestive tract, gastrointestinal tract, liver, bladder, prostate, uterus, ovary and breast, and often show an aggressive clinical course (2-4). Of these, esophageal carcinosarcoma (ECS) is a rare malignant neoplasm that accounts for 0.5-2.8% of all esophageal malignancies (5). ECS is usually composed of invasive and/or *in situ* squamous cell carcinoma and sarcoma-like cells (6). However, ECS is generally thought to be derived from a single-cell clone of epithelial cells, with the sarcoma-like cells emerging as a subclone from the carcinoma cells through mesenchymal metaplasia (7).

Regarding patient prognosis, investigators have suggested that ECS often presents as a polypoid lesion protruding into the esophageal lumen, and is detected at a relatively earlier stage than pure squamous cell carcinoma, leading to a comparatively good prognosis (8). Other reports, however, have indicated similar 5-year survival rates in patients with pure esophageal squamous cell carcinoma (ESCC) and ECS (5).

Radical esophagectomy with lymph node dissection is currently the standard therapy for ECS patients, and systemic adjuvant therapies may be considered in progressive cases, as for ESCC patients. However, chemotherapies, which generally involve the same regimen as for ESCC, are usually insufficient

to control the growth of ECS at metastatic sites (9). Although lymph node metastases occur in ~50% of ECS cases (10), sarcoma-predominant components preferentially metastasize to distant organs or the peritoneum, and rarely result in lymph node metastasis (11). These sarcomatous components at metastatic sites may define the prognosis of patients with ECS, since unlike carcinomas, most soft tissue sarcomas are notoriously resistant to standard chemotherapies (12).

Overexpression of receptor tyrosine kinases (RTKs) has recently been reported in various types of malignant tumors, and these represent attractive molecular targets for alternative therapies using effective and safe selective inhibitors. RTKs are key molecules in normal cellular differentiation and proliferation, but are commonly deregulated in various types of human cancers. RTK inhibitors have recently been reported to be effective in the treatment of several tumor types, including breast, lung and colon cancer, gastrointestinal stromal tumors and renal cancers (13,14). RTKs also play an important role in ESCC, and certain RTK inhibitors may represent useful therapeutic strategies for esophageal cancer (15). However, no studies have analyzed the expression of RTKs in ECS, and their status in these tumors thus remains poorly understood.

We previously reported variable histological and immunohistochemical phenotypes of the sarcomatous components in ECS cases (16), suggesting that the expression of RTKs in ECS may differ from that in ESCC. The optimal chemotherapeutic approach for ESC might thus also differ from the standard therapy for ESCC.

We examined for the first time the expression patterns and genetic alterations of various RTKs in each squamous cell and sarcomatous component of ECS, and provides some rationale for the administration of molecular-targeted drugs for ECS.

Materials and methods

Patient characteristics and tissue samples. This study included 20 cases of ECS as described previously (16), and 1 additional case, making a total of 21 patients diagnosed with ECS at Gunma Prefectural Cancer Center, Gunma University Hospital, Niigata University Hospital and Jichi Medical School Hospital. These patients included 20 surgical cases and 1 autopsy case. All the patients were males, with a mean age of 67 years (range 51-81 years). The surgical specimens were fixed with 10% formalin and embedded in paraffin, and 3-μm sections were prepared and stained with hematoxylin and eosin. The diagnosis of ECS was confirmed histologically by two pathologists. Clinical information was obtained from medical records in all cases.

Immunohistochemistry (IHC). Formalin-fixed paraffin-embedded tissue specimens for each patient were cut into 3-μm sections and used for IHC. The antibodies used in this study, as well as the dilution and antigen-retrieval method for each antibody are listed in Table I.

The cellular differentiation of the mesenchymal component in each ECS was characterized immunohistochemically using the following antibodies: smooth muscle actin (α-SMA) and desmin as markers of muscle differentiation; S100 protein as a marker of neural differentiation or chondroid differentiation and vimentin as a marker of mesenchymal differentiation.

The expression levels of various RTKs [KIT, platelet-derived growth factor receptor (PDGFR)A, PDGFRB, MET, epidermal growth factor receptor (EGFR) and HER-2] were also examined by IHC in each epithelial and mesenchymal component of the 21 ECSs.

Tissue sections were deparaffinized with xylene and rehydrated through decreasing concentrations of alcohol. Endogenous peroxidase activity was blocked by immersion with 0.3% hydrogen peroxide in absolute methanol for 30 min. After antigen retrieval, or without antigen retrieval, the primary antibody was applied and incubated overnight at 4˚C in a high-humidity chamber. EnVision+ (Dako, Glostrup, Denmark) was used with a secondary antibody for 60 min at room temperature. The slides were incubated in 3'-diaminobenzidine tetrahydrochloride solution, counterstained with hematoxylin and mounted. Serial sections of selected tissue samples were immunostained in the absence of the primary antibody, as a negative control.

The expression levels of the RTKs were evaluated separately in the epithelial and mesenchymal components in each case. Immunoreactivity for each antibody was quantitated by scoring the intensity of staining (0, negative; 1+, weak; 2+, moderate; 3+, strong), and the percentage of positive cells was calculated for each section without reference to any clinical information. IHC was judged to be positive when ≥5% of the tumor cells were stained moderately (2+) to strongly (3^+).

Ki-67 expression was also evaluated to assess the proportion of proliferating cells. The percentage of Ki67-positive nuclei among 1,000 tumor cells was evaluated and defined as the Ki67-labeling index (LI) in each epithelial and mesenchymal component.

Mutational analysis of c-kit, PDGFRA, c-met and EGFR genes. Mutational analysis of previously reported hotspots for each RTK gene was performed for all cases that were positive for each RTK by IHC. Mutation analysis was performed as previously described (17). Genomic DNA was extracted from formalin-fixed, paraffin-embedded tumor tissues. The epithelial and mesenchymal components were dissected and subjected to proteinase K treatment in an extraction buffer (10 mmol/l Tris HCl, pH 8.0; 1 mmol/l EDTA; and 1% Tween-20) and incubated overnight at 62˚C. Exons 9, 11, 13 and 17 of *c-kit*, exons 12 and 18 of *PDGFRA*, exon 14 of *c-met* and exons 19-21 of *EGFR*, which were identified as mutational hot spots in previous reports, were amplified by polymerase chain reaction. The forward and reverse oligonucleotide primers used in this study are listed in Table II. Nested PCR amplification was carried out for the *EGFR* gene. Each of the amplified fragments was purified from a polyacrylamide gel, and direct sequencing was carried out using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130 DNA Sequencer (Applied Biosystems). All sequencing reactions were carried out in forward and reverse directions.

Fluorescence in situ hybridization (FISH). FISH analysis was performed for all tumors positively stained with the RTK antibodies to define the status of the *c-kit*, *PDGFRA*, *c-met* and *EGFR* genes. The following DNA probe mixtures were used: *c-kit* (BAC clone RP11-586A2 SpectrumGreen)/CEP4

Table I. Antibodies used for immunohistochemistry.

Antibody	Clone	Source	Dilution	Antigen retrieval
KIT	mAb, Y145	Epitomics	1:100	MW
PDGFRA	pAb	Santa Cruz Biotechnology	1:200	AC
PDGFRB	pAb	Santa Cruz Biotechnology	1:400	AC
MET	pAb	Santa Cruz Biotechnology	1:200	AC
EGFR	mAb, 31G7	Invitrogen	1:200	Trypsin
HER-2	pAb	Dako	1:100	MW
Vimentin	mAb, V9	Dako	1:10	-
Smooth muscle actin	mAb, 1A4	Dako	1:160	-
Desmin	mAb, D33	Dako	1:50	-
S100	pAb	Dako	1:800	-
Ki-67	mAb, MIB-1	Dako	1:40	Trypsin/MW

Epitomics, Burlingame, CA, USA; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; Invitrogen, Carlsbad, CA, USA; Dako, Glostrup, Denmark. MW, microwave; AC, autoclaving.

Table II. Oligonucleotide primers used for direct sequence analysis.

Gene and exons	Sequence	Fragment size (bp)	Annealing temperature (˚C)
c-kit			
Exon 9	Forward 5'-ATGCTCTGCTTCTGTACTGCC-3'	238	55
	Reverse 5'-CAGAGCCTAAACATCCCCTTA-3'		
Exon 11	Forward 5'-CCAGAGTGCTCTAATGACTG-3'	236	53
	Reverse 5'-ACCCAAAAAGGTGACATGGA-3'		
Exon 13	Forward 5'-CATCAGTTTGCCAGTTGTGC-3'	174	55
	Reverse 5'-ACACGGCTTTACCTCCAAATG-3'		
Exon 17	Forward 5'-TGTATTCACAGAGACTTGGC-3'	218	55
	Reverse 5'-GGATTTACATTATGAAAGTCACAGG-3'		
PDGFRA			
Exon 12	Forward 5'-TCCAGTCACTGTGCTGCTTC-3'	260	56
	Reverse 5'-GCAAGGGAAAAGGGAGTCTT-3'		
Exon 18	Forward 5'-ACCATGGATCAGCCAGTCTT-3'	255	56
	Reverse 5'-AAGTGTGGGAGGATGAGCCTG-3'		
c-met			
Exon 14	Forward 5'-TTCTGGGCACTGGGTCAAAGT-3'	281	58
	Reverse 5'-AATGTCACAACCCACTGAGGT-3'		
EGFR			
Exon 19	Forward 5'-GCAATATCAGCCTTAGGTGCGGCTC-3'	372	58
	Reverse 5'-CATAGAAAGTGAACATTTAGGATGTG-3'		
Exon 19 (nested PCR)	Forward 5'-CCTTAGGTGCGGCTCCACAGC-3'	349	58
	Reverse 5'-CATTTAGGATGTGGAGATGAGC-3'		
Exon 20	Forward 5'-CCATGAGTACGTATTTTGAAACTC-3'	408	58
	Reverse 5'-CATATCCCCATGGCAAACTCTTGC-3'		
Exon 20 (nested PCR)	Forward 5'-GAAACTCAAGATCGCATTCATGC-3'	379	58
	Reverse 5'-GCAAACTCTTGCTATCCCAGGAG-3'		
Exon 21	Forward 5'-CTAACGTTCGCCAGCCATAAGTCC-3'	415	58
	Reverse 5'-GCTGCGAGCTCACCCAGAATGTCTGG-3'		
Exon 21 (nested PCR)	Forward 5'-CAGCCATAAGTCCTCGACGTGG-3'	374	58
	Reverse 5'-CATCCTCCCCTGCATGTGTTAAAC-3'		

(BAC clone RP11-217B22 SpectrumOrange), *PDGFRA* (BAC clone RP11-231C18 SpectrumGreen)/CEP4 (BAC clone RP11-217B22 SpectrumOrange), *c-met* (BAC clone RP11-163C9 SpectrumOrange)/CEP7 (BAC clone RP11-90C3 SpectrumGreen) (Chromosome Science Labo, Sapporo, Japan) and *EGFR* (LSI EGFR SpectrumOrange)/CEP7 (SpectrumGreen) (Vysis; Abbott Laboratories, Downers Grove, IL, USA).

Representative areas of the tissue sections that showed positive immunostaining for each RTK were selected and trimmed for FISH. Formalin-fixed paraffin-embedded tissue was prepared in serial 6-μm sections. After dewaxing in xylene and dehydration in 100% ethanol, sections were immersed in 0.2 N HCl for 20 min and incubated in 1 M NaSCN pretreatment solution (Vysis) for 30 min at 80˚C. Sections were digested with protease solution (Vysis) for 60 min at 37˚C and fixed with 10% formalin for 10 min, denatured at 72˚C for 5 min in 70% formamide/2X standard saline citrate (SSC), and dehydrated through a series of graded ethanols. A volume of 10 μl of the denatured DNA probe mixture was applied to the hybridization area and covered with a glass coverslip. After microwaving for 60 min at 40˚C, the slides were hybridized at 37˚C for 48 h. Sections were washed in post-hybridization wash solution (2X SSC, 0.3% NP-40) at 73˚C for 2 min and counterstained with 4,6-diamidino-2-phenylindole (DAPI).

The signals were counted in at least 50 nuclei/slide under x1,000 magnification in each selected area, and the target gene/CEP ratio was calculated. The cytogenetic patterns were classified according to the criteria of Cappuzzo *et al* (18): high polysomy (≥4 copies in ≥40% cells) and gene amplification (defined by presence of tight gene clusters, a gene/chromosome ratio ≥2, or ≥15 copies/cell in ≥10% of analyzed cells) were considered as FISH-positive. Disomy (≤2 copies in >90% of cells); trisomy (≤2 copies in ≥40% of cells, ≥4 copies in <10% of cells) and low polysomy (≥4 copies in 10-40% of cells) were considered as FISH-negative.

Statistical analysis. The significance of differences was analyzed by applying the χ^2 test or Fisher's exact test. Differences were considered significant when the P-value was <0.05.

Results

Clinicopathological characteristics. All cases were morphologically defined as protruded type, type 1 according to the Japanese macroscopic classification. Four cases also had ulcerative or infiltrative lesions.

All tumors consisted microscopically of both epithelial and mesenchymal components, although the proportions of the components varied among the cases. The epithelial components in all cases were squamous cell carcinoma. Histologically, the mesenchymal components were malignant fibrous histiocytoma (MFH)-like in 3 (Fig. 1A), leiomyosarcoma-like in 2 (Fig. 1B) and chondrosarcoma-like in 1 case (Fig. 1C). The remaining 15 cases were composed of pleomorphic spindle cells (Fig. 1D).

Immunohistochemical expression of mesenchymal markers and Ki-67. Some of the results were previously reported (16). All histologically classified mesenchymal components were immunohistochemically positive for more than one mesenchymal marker. Of the 21 ECSs, vimentin, α-SMA, desmin and S-100 were expressed in the mesenchymal component in 19 (90.5%), 16 (76.2%), 0 and 3 cases (14.3%), respectively. The corresponding values in the epithelial component were 1 (4.8%), 1 (4.8%), 0 and 1 (4.8%), respectively (Table III).

The Ki-67 LI in the epithelial component ranged from 15.6 to 58.4%, while that in the mesenchymal component ranged from 19.5 to 61.4% (Table III). In 7 cases (case nos. 3, 4, 6, 8, 16, 19 and 20), the Ki-67 LI of the mesenchymal component was ≥10% higher than that of the epithelial component, whereas the Ki-67 LI of the mesenchymal component was ≥10% lower in case nos. 11 and 21. There was no significant difference in average Ki-67 LI between the epithelial and mesenchymal components.

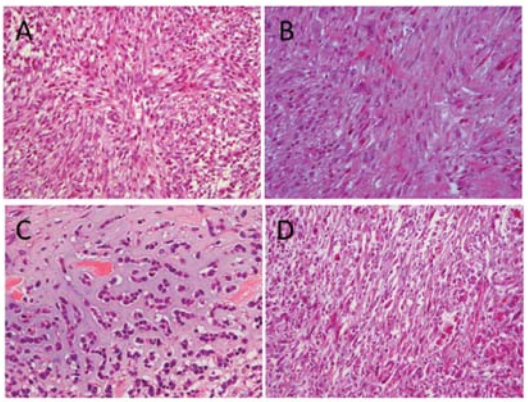

Figure 1. Histological examination of the mesenchymal components in esophageal carcinosarcoma revealed (A) malignant fibrous histiocytoma-like, (B) leiomyosarcoma-like, (C) chondrosarcoma-like and (D) pleomorphic spindle-cell features.

Immunohistochemical expression of receptor tyrosine kinases. The immunohistochemical expression of the various RTKs is summarized in Table III. Representative IHC results for MET and EGFR are shown in Fig. 2.

Normal esophageal epithelium adjacent to the tumor tissue was negative for all RTKs and was scored as 0. Among the 21 ECSs, KIT overexpression was observed in 3 (14.3%) cases, including in the mesenchymal component in 2 cases (case no. 8 and 12) and the epithelial component in 1 case (case no. 9). PDGFRA overexpression was detected in 3 (14.3%), including 2 in the mesenchymal component (case nos. 14 and 15) and one in the epithelial component (case no. 20). None of the 21 tumors showed PDGFRB overexpression. Overexpression of MET was detected in 16 of the 21 cases (76.2%). This was limited to the mesenchymal components in 5 cases (case nos. 4, 8, 10, 14 and 16), to the epithelial component in 4 cases (case nos. 9, 17, 19 and 20) and occurred in both the epithelial and mesenchymal components in 7 cases (case nos. 1, 2, 3, 5, 6, 11 and 12). EGFR overexpression was detected in 19 of 21 cases (90.5%), restricted to the mesenchymal component in 6 cases (case nos. 4, 7, 8, 14, 15 and 16), the epithelial component in 8 cases (case nos. 1, 3, 9, 11, 17, 18, 19 and 21), and to both the epithelial and mesenchymal components in 5 cases (case nos. 2, 5, 6, 10 and 12). HER-2 overexpression was observed in the epithelial component in 2 cases (9.5%).

Among the 21 ECSs, MET and/or EGFR were co-expressed in 15 cases (71.4%). Of these, 4 showed co-overexpression of MET and EGFR in both the epithelial and mesenchymal components (case nos. 2, 5, 6 and 12), 6 only in the epithelial component (case nos. 1, 3, 9, 11, 17 and 19) and 5 only in the mesenchymal component (case nos. 4, 8, 10, 14 and 16).

There was no correlation between overexpression of any RTK with clinicopathological factors.

Table III. Overexpression of receptor tyrosine kinases and mesenchymal markers in epithelial and mesenchymal components of the esophageal carcinosarcoma cases.

Case no.		Expression of proteins by immunohistochemistry								Ki-67 LI (%)
		KIT	PDGFRA	PDGFRB	MET	EGFR	HER-2	Vimentin	Mesenchymal markers	
1	E	-	-	-	+	+	-	-	-	41.3
	M	-	-	-	+	-	-	+	α-SMA	34.2
2	E	-	-	-	+	+	-	-	-	50.9
	M	-	-	-	+	+	-	+	-	42.7
3	E	-	-	-	+	+	-	-	-	30.9
	M	-	-	-	+	-	-	+	α-SMA	45.7
4	E	-	-	-	-	-	-	-	-	46.2
	M	-	-	-	+	+	-	+	α-SMA	58.5
5	E	-	-	-	+	+	+	-	-	45.0
	M	-	-	-	+	+	-	+	α-SMA	46.9
6	E	-	-	-	+	+	-	-	-	15.6
	M	-	-	-	+	+	-	+	-	38.5
7	E	-	-	-	-	-	-	-	-	19.8
	M	-	-	-	-	+	-	+	α-SMA	23.8
8	E	-	-	-	-	-	-	-	-	22.5
	M	+	-	-	+	+	-	+	α-SMA, S100	32.8
9	E	+	-	-	+	+	-	-	S100	44.0
	M	-	-	-	-	-	-	+	α-SMA	51.6
10	E	-	-	-	-	+	-	-	-	36.2
	M	-	-	-	+	+	-	+	α-SMA	39.4
11	E	-	-	-	+	+	-	-	-	35.3
	M	-	-	-	+	-	-	+	α-SMA	19.5
12	E	-	-	-	+	+	+	-	α-SMA	58.4
	M	+	-	-	+	+	-	+	α-SMA, S100	61.4
13	E	-	-	-	-	-	-	-	-	39.1
	M	-	-	-	-	-	-	+	-	42.4
14	E	-	-	-	-	-	-	-	-	30.8
	M	-	+	-	+	+	-	-	α-SMA, S100	35.7
15	E	-	-	-	-	-	-	-	-	37.4
	M	-	+	-	-	+	-	+	-	43.9
16	E	-	-	-	-	-	-	-	-	14.4
	M	-	-	-	+	+	-	+	α-SMA	31.6
17	E	-	-	-	+	+	-	-	-	40.3
	M	-	-	-	-	-	-	+	-	40.6
18	E	-	-	-	-	+	-	-	-	41.5
	M	-	-	-	-	-	-	+	α-SMA	49.4
19	E	-	-	-	+	+	-	-	-	37.9
	M	-	-	-	-	-	-	-	α-SMA	59.6
20	E	-	+	-	+	-	-	+	-	23.6
	M	-	-	-	-	-	-	+	α-SMA	36.8
21	E	-	-	-	-	+	-	-	-	40.9
	M	-	-	-	-	-	-	+	α-SMA	20.1
Total		3 (14.3%)	3 (14.3%)	0 (0%)	16 (76.2%)	19 (90.5%)	2 (9.5%)	19 (90.5%)	16 (76.2%)	

E, epithelial component; M, mesenchymal component; LI, labeling index.

Mutational analysis of c-kit, PDGFRα, c-met and EGFR genes. No mutations were found in any of the analyzed exons of the *c-kit*, *PDGFRα* and *c-met* genes.

The same missense point mutation at codon 790 (ACG to GCG) of the *EGFR* gene exon 20 was found in 2 of the 19 EGFR-positive ESCs (case nos. 15 and 18), resulting in

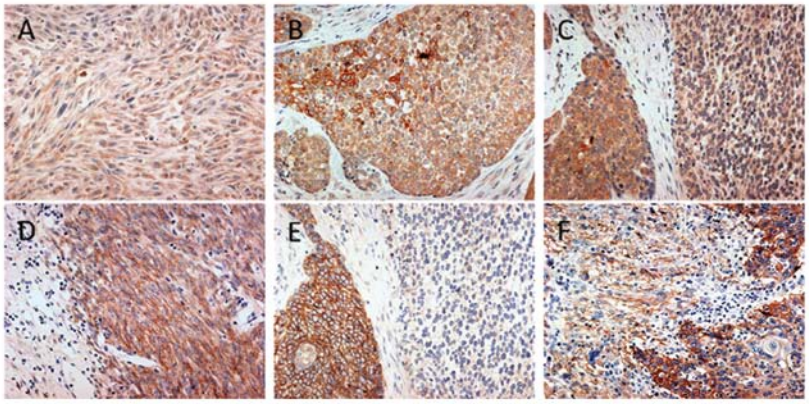

Figure 2. Representative immunohistochemical results for MET and EGFR. MET expression was observed in (A) the mesenchymal component, (B) the epithelial component, and (C) both the mesenchymal and epithelial components. EGFR expression was observed in (D) the mesenchymal component, (E) the epithelial component, and (F) both the mesenchymal and epithelial components.

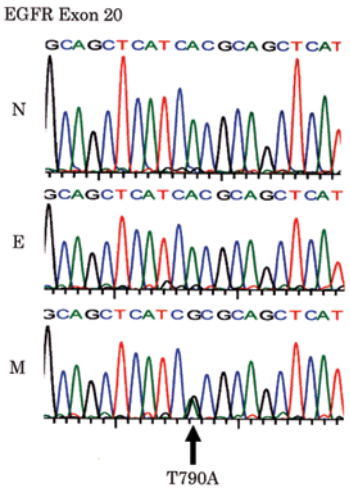

Figure 3. Direct sequence analysis indicated a threonine to alanine amino acid substitution at codon 790 (T790A) as a result of an A-G substitution at position 2368 in exon 20 (arrow head, mutation point) only in the mesenchymal component. This T790A mutation was observed in 2 cases of esophageal carcinosarcoma. N, normal tissue; E, epithelial component; M, mesenchymal component.

substitution of threonine by alanine (T790A). These missense mutations were only observed in the mesenchymal components in both cases (Fig. 3). They were not detected in normal squamous epithelium from the same patients, and were therefore considered to be somatic mutations.

Status of c-kit, PDGFRα, c-met and EGFR genes by FISH analysis. Representative results of FISH analysis of the *EGFR* gene in ECS tissue samples are shown in Fig. 4. Gene status by FISH analysis is shown in Table IV. Among the 3 cases with KIT overexpression, case no. 12 was FISH-positive (high polysomy) and the remaining 2 cases were FISH-negative (low polysomy in case no. 9 and disomy in case no. 8). All 3 patients with PDGFRA overexpression were FISH-negative (trisomy in case no. 15 and disomy in case no. 14 and 20). In the 16 cases with MET overexpression, 3 cases were FISH-positive (high polysomy in case nos. 5, 6 and 11), and the rest were FISH-negative (low polysomy in case nos. 9, 16, 19 and 20, trisomy in case nos. 1, 3, 12 and 17, and disomy in case nos. 2, 4, 8, 10 and 14). Among the 19 cases with EGFR overexpression, 10 were FISH-positive (gene amplification in case nos. 2 and 9, and high

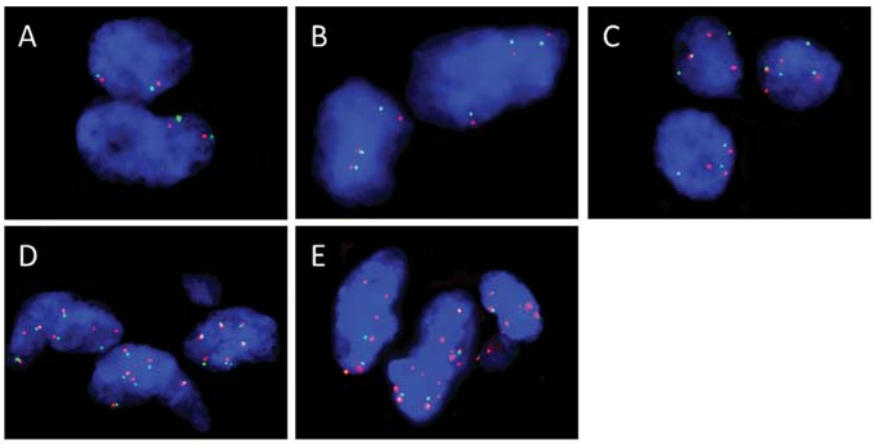

Figure 4. *EGFR* gene alteration was detected by fluorescence *in situ* hybridization using LSI EGFR/CEP7 Dual Color Probe containing centromere chromosome 7 (SpectrumGreen) and EGFR (SpectrumOrange). (A) Two orange and green signals were observed in tumor cell nuclei corresponding to the *EGFR* gene and CEP7, respectively (disomy). (B) Trisomy of chromosome 7. (C) Low polysomy of chromosome 7. (D) High polysomy of chromosome 7 with a ratio of ~1; more than four chromosomal signals were associated with a similar number of *EGFR* gene signals (red). (E) Amplification of the *EGFR* gene: red/green (EGFR/CEP7) signal ratio >2. Gene clusters are observed.

Table IV. Gene status of receptor tyrosine kinases by fluorescence *in situ* hybridization.

	Gene status	*c-kit* (n=3)	*PDGFRA* (n=3)	*c-met* (n=16)	*EGFR* (n=19)
FISH-positive	Gene amplification	0	0	0	2
	High polysomy	1	0	3	8
FISH-negative	Low polysomy	1	0	4	2
	Trisomy	0	1	4	3
	Disomy	1	2	5	4

polysomy in case nos. 3-6, 12, 16, 17 and 19), and the rest were FISH-negative (low polysomy in case nos. 7 and 21, trisomy in case nos. 1, 10 and 11, and disomy in case nos. 8, 14, 15 and 18).

There was no significant correlation between the results of FISH and any clinicopathological factors.

Discussion

We previously demonstrated variable histological and immunohistochemical phenotypes in the mesenchymal components of ECSs, including MFH-like, leiomyosarcoma-like, and chondrosarcoma-like features. The proliferative activity of tumor cells, assessed by Ki-67 LI, also varied between cases, with the mesenchymal component tending to show higher proliferation than the epithelial component in each case. These results seem to be compatible with the idea that the mesenchymal component develops by transition from squamous cell differentiation to mesenchymal differentiation, and plays an important role in tumor progression.

In addition to these findings, the present study also demonstrated that various RTKs were overexpressed in tumor cells in ECS, with MET and EGFR especially being highly co-expressed in most ECSs.

Overexpression of MET and/or alteration of the *c-met* gene has been reported in a wide variety of tumors, including carcinomas and sarcomas (19). The MET oncogene can be activated by overexpression, gene rearrangements, or mutations in tumor cells, resulting in tumor development and progression (19,20).

Previous studies have reported overexpression of MET in up to 54% of esophageal adenocarcinomas (EAs) (21) and 92% of ESCCs (22), and expression levels are thought to correlate with tumor development, progression, and prognosis in patients with EA and ESCC (21-23). However, there have been no reports of MET expression in ECS. The present study demonstrated overexpression of MET in 76.2% of ECSs, an intermediate percentage between EA and ESCC. Three of those cases showed increased copy numbers of the *c-met* gene by FISH analysis.

EGFR overexpression has been reported in 33-50% of ESCCs (24,25) and 55% of EAs (26). Amplification of the *EGFR* gene has also been reported in ~30% of ESCCs and 6-11% of EA cases (25,27). The rate of EGFR overexpression in ECS in the present study was much higher (19 of 21 cases, 90.5%), and associated with amplification or high polysomy of the *EGFR* gene. Furthermore, 2 of the 19 cases had the same missense point mutation (T790A) in *EGFR* exon 20 restricted to the mesenchymal component of ECS. Codon 790 in the *EGFR* gene is a mutational hotspot for secondary resistance to gefitinib in non-small cell lung cancer (28).

EGF/EGFR signaling pathways have recently been reported to induce cancer cell epithelial-mesenchymal transition (EMT) via STAT3-mediated TWIST gene expression (29), upregulation of Snail (30) and loss of E-cadherin and increased invasion of cancer cells (31). Snail-associated EMT has been reported to promote tumor invasiveness, migration and proliferative activity in ESCC (32). Dysregulated MET/HGF signaling is also correlated with tumor proliferation and survival, increased cell motility and migration, tumor invasion and metastasis (33). MET/HGF signaling recruits and activates c-Src, which subsequently phosphorylates E-cadherin resulting in Numb dissociation from phosphorylated E-cadherin, and several downstream signaling pathways participate in the reduction of cell-cell adhesion, cell proliferation and cell migration, i.e., EMT (34).

The present study identified a high frequency of MET and EGFR co-expression in ECS (15 of 21 cases, 71%). A recent experimental study indicated that mutant p53 and EGFR expression potentiated HGF/MET signaling (35). The present and previous results suggest that co-expression of MET and EGFR may play a key role in mesenchymal sarcomatous metaplasia of squamous cell carcinoma through mechanisms such as EMT in other type of carcinomas, with subsequent tumor progression.

Regarding chemotherapy for esophageal cancer, cisplatin/5-fluorouracil (5-FU) is the accepted standard treatment. The effectiveness of combination chemotherapies such as 5-FU/nedaplatin (a third-generation platinum), docetaxel/cisplatin/5-FU, and paclitaxel/cisplatin/5-FU has been reported in recent years (36). Furthermore, several molecular-targeted therapies have been assessed for advanced esophageal cancer, including monoclonal antibodies and signal transduction/tyrosine kinase inhibitors for EGFR, HER2/neu receptor, vascular endothelial growth factor ligand, cyclooxygenase-2 inhibitors and other novel drugs (36-39). Of these trials, gefitinib appeared to have no activity in EA, whereas limited activity was observed in patients with squamous cell carcinoma (37). Phase II trials of erlotinib reported activity in ESCC (38) and phase II trials of combinations of EGFR-targeted monoclonal antibodies with chemotherapy, such as FOLFIRI (leucovorin/5-FU/irinotecan) with cetuximab, are now underway (39).

However, metastatic ECSs have been reported to respond poorly to conventional chemotherapy and radiation, probably due to the sarcomatous differentiation of tumor cells. New treatment options for ECS patients, therefore, need to be investigated. The results of this study suggest that molecular-targeting therapies directed to MET and EGFR may be effective in inhibiting the growth or progression of the epithelial and/or mesenchymal components of ECS. Further investigations are warranted to

establish the rationale for the use of such molecular-targeting therapies for this highly malignant cancer type.

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