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Overexpression and gene amplification of both *ERBB2* and *EGFR* in an esophageal squamous cell carcinoma revealed by fluorescence *in situ* hybridization, multiplex ligation-dependent probe amplification and immunohistochemistry

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Running title: Gene amplification in esophageal cancer

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Abstract

***EGFR* and *ERBB*2 belong to the *EGFR* gene family. In esophageal squamous cell carcinomas (SCCs), amplification of *EGFR* or *ERBB*2 is usually mutually exclusive. *EGFR* amplification occurs in approximately 15% of SCCs, *ERBB*2 occurs in less than 5%. Here, we report the co-amplification of *EGFR* and *ERBB2* in an ulcerative and infiltrating-type SCC that measured approximately 4.2x2.7x1.2 cm with a superficial lesion occurring in the thoracic esophagus of a 72-year-old man. Multiplex ligation-dependent probe amplification using representative tumor sections showed gain of *CCND1* and coincident amplification of *ERBB2* or *EGFR* or neither. Immunohistochemistry and fluorescence *in situ* hybridization revealed that the tumor comprised three cancer-cell populations: well-differentiated SCC with high-level *ERBB2* amplification and ERBB2 overexpression, more infiltrative poorly-differentiated SCC with high-level *EGFR* amplification and EGFR overexpression, and poorly-differentiated SCC lacking any *ERBB2* or *EGFR* abnormality. These three populations each had low-level *CCND1* amplification and nuclear cyclin D1 overexpression. This histological topology and gene amplification combinations suggested that genetic instability first produced *CCND1* amplification, and then *ERBB2* or *EGFR* gene amplification occurred. It is further speculated that during cancer progression and clonal selection indecisive predominance of either clone caused the rare co-amplification of *ERBB2* and *EGFR* in a single chimeric tumor.**

Introduction

Esophageal cancer ranks sixth among all cancers in mortality and is the eighth most common cancer worldwide. Squamous cell carcinoma (SCC) is the predominant histologic type of esophageal cancer worldwide.[1] Despite improvements in surgical techniques, peri-operative management, and combination therapies involving surgery combined with chemotherapy and/or radiotherapy, the prognosis of advanced-stage esophageal SCC at remains poor. Therefore, novel therapies (e.g., molecular-targeted therapy) are very much needed for patients with esophageal SCC.[2] Currently, the most promising targets for molecular therapy are amplified *ERBB2* and *EGFR*, which belong to the *EGFR* gene family and encode type I receptor tyrosine kinases (RTKs). Two categories of therapies targeting EGFR family members have been or are in clinical development: 1) small-molecule inhibitors (e.g., gefitinib, erlotinib, and lapatinib) of EGFR family-related tyrosine kinases and 2) humanized antibodies against ERBB2 or EGFR (trastuzumab and cetuximab, respectively). There has been some discrepancy in findings regarding frequency of *EGFR* amplification; nevertheless, in our previous fluorescence *in situ* hybridization (FISH) study, the frequency of high-level *EGFR* amplification was 15%,[3] and a more recent study using FISH reported the same figure.[4] The frequencies of *ERBB2* amplification previously reported were much lower and

ranged from 2% to 5%.[4, 5] Recent comprehensive and semi-comprehensive studies examining RTK genes in advanced cancers indicate that amplification of an individual RTK gene is usually exclusive; that is, each tumor usually has only one amplified RTK gene.[1, 2]

Cyclin D1 expressed during the G1-S phase transition of the cell cycle can bind to retinoblastoma whose inactivation untethers E2F from inhibitory constraints and thereby allows activation of genes required for DNA replication.[6] Cyclin D1 overexpression is significantly associated with cyclin D1 gene (*CCND1*) amplification, which was found to occur in 42%[4] or 53% [7] of esophageal SCCs based on FISH analyses.

Multiplex ligation-dependent probe amplification (MLPA) is a new, high-resolution method for detecting numerous copy number variations throughout an individual's genome; MLPA assays require only one reaction tube and small amounts of DNA extracted from formalin-fixed paraffin-embedded clinical samples.[8, 9] Cancer progression occurs via a process in which different clones and subclones are produced by genetic instability and simultaneously subjected to Darwinian selection. Here, we present a case of esophageal SCC in which the clonal profile of *ERBB2, EGFR*, and *CCND1* amplification could be accurately and precisely determined via

immunohistochemistry (IHC), MLPA, and FISH.

Clinical summary

A 72-year-old man who had received medical care for hepatitis C presented to the Department of Gastroenterologic Surgery, Kanazawa University in September 2012 describing a sense of constriction in his throat. Upper gastrointestinal examinations revealed an advanced SCC in the mid-thoracic esophagus. In January 2013, after one course of preoperative chemotherapy (a regimen of docetaxel, cisplatin, and fluorouracil), thoracoscopic esophagectomy with cervical, mediastinal, and abdominal lymph node dissection and gastric tube reconstruction under laparotomy were performed. The postoperative course was uneventful, and recurrence was not found in February, 2015. Our laboratory study of this case was approved by the Institutional Review Board at Kanazawa University, and written informed consent was obtained from the patient.

Pathological findings

Surgical specimens

An ulcerative and infiltrative tumor measuring approximately 4.2×2.7×2.0 cm with a superficial lesion extending 5.2 cm in length was found (Fig. 1). Histologically, the presented tumor broadly divided two parts, well- and poorly- differentiated SCC: the well-differentiated SCC forming cancer pearls (Fig. 2a) was predominant and poorly differentiated SCC that occupied the center of the tumor and penetrated the adventitia (Fig. 2b). This poorly differentiated SCC contained irregular-shaped necrosis or fibrosis occasionally accompanied by foreign-body reaction corresponding to a Grade 1a therapeutic effect (Fig. 2c). Metastases of poorly differentiated SCC were evident in four regional lymph nodes. Pathological stage was ypIIIB according to the American Joint Committee on Cancer (AJCC) criteria. [10]

MLPA

The SALSA MLPA KIT P175-A2 Tumor-Gain (MRC-Holland, Amsterdam, The Netherlands) was used for MLPA analysis; this kit contains two or three probes for each of 24 genes, including *ERBB2, EGFR,* and *CCND1*. A representative tumor area approximately 3.0 cm^2 was identified in consecutive 6-μm-thick sections that were adjacent hematoxylin and eosin (HE)-stained sections. DNA was extracted manually

according to the manufacturer's protocol. Data analysis was performed with Coffalyser MLPA-DAT software (version 9.4, MRC-Holland) to generate normalized peak values. As previously established, peak values below 0.7 were defined as "lost", those between 0.7 and 1.3 as "normal", those between 1.3 and 2.0 as "gain", and values >2.0 were defined as "amplified".[11]

The results showed that *ERBB2* and *EGFR* were amplified; notably, all *CCND1* values were categorized as "gain". Values for the other 21 genes were within the normal range (Table 1).

Methods for IHC and FISH

Antibodies against the internal domain of human ERBB2 (polyclonal, Nichirei, Tokyo, Japan; working dilution of 1:100), the external domain of human EGFR (Novocastra Lab, Newcastle, UK; working dilution, 1:20), or human cyclin D1 (rabbit monoclonal, SP 4, Nichirei, ready-to-use) were used. Antibodies were visualized by avidin-biotin binding to peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark). For evaluation of positive ERBB2 or EGFR staining, a four-tier system (0, 1+, 2+, or 3+) based on the criteria recommended by Dako for the HercepTest™ was

used, except that the quantity of positive cells was not considered. For evaluation of cyclin D1 staining, only nuclear immunostaining significantly higher than that seen in control cells of normal esophageal mucosa were considered to be positive.

FISH probes for *ERBB2* (RP11-62N23), *EGFR* (RP11-339F13), and *CCND1* (RP11-775J10) were acquired from BACPAC Resources (Oakland, CA, USA) and labeled with SpectrumOrange^{TM} or SpectrumGeen^{TM} with a nick translation kit (Abbott Laboratories, Abbott Park, IL, USA). For detection of gene amplification, SpectrumGreen^{TM}-labeled pericentromeric probes (Abbott) specific to chromosome 7, 11, or 17 on which the respective gene was located were co-hybridized with the appropriate gene-specific probe to standardize for chromosome number. In order to determine the distribution of *ERBB2*-amplified cells and *EGFR*-amplified cells, and co-localization of *CCND1* and *ERBB* or *EGFR,* dual–color FISH using two probes labeled with different fluorescences was also performed.

Results of IHC and FISH

Based on IHC, cells with 3+ or 2+ ERBB2 staining were found in well-differentiated SCC (Fig. 2a) and mucosal cancer in the tumor margin (Fig.2d); in contrast, the center of the tumor that corresponded to poorly differentiated SCC was predominantly

ERBB2-negative and populated with mostly EGFR 3+ cells. A few nests that were both ERBB2-negative and EGFR-negative were also found in the center of the tumor (Fig. 2b). Small foci of EGFR immunostaining were present in spots throughout the tumor; however, staining intensities in these foci did not exceed 2+. Cancer-cell nuclei IHC-positive for cyclin D1 were distributed evenly among ERBB2-positive cells, EGFR-positive cells, and double-negative cells (Fig. 2e).

Based on FISH analysis, the IHC ERBB2-positive cells had one to four tightly clustered *ERBB2* FISH signals, suggesting that amplicons existed in homogeneously staining regions (HSR) [12] (Fig. 3a). Each poorly differentiated SCC cells with 3+ EGFR IHC staining had one or two large clustered *EGFR* FISH signals; this pattern also suggested that amplification had occurred in HSRs (Fig. 3b). No gene amplification was evident in cells with 1+ or 2+EGFR-immunostaining. *ERBB2*-positive cells and *EGFR*-positive cells were mutually exclusive, and there was no single cell with both genes amplified even in areas where the two types of cancer cells were directly apposed (Fig. 3c).

Most cancer-cell nuclei had one to five additional *CCND1* signals relative to centromere 11 signals, and the ratios of *CCND1* copy number to centromere 11 copy number ranged from 2.4 to 2.6, suggesting low-level *CCND1* amplification (Fig. 3d).

Dual-color FISH showed that *ERBB2*-amplified cells (Fig. 3e) and *EGFR*-amplified cells (Fig. 3f) each had co-amplification of *CCND1*. Metastatic cancer in the lymph nodes was cyclin D1-positive, but ERBB2- and EGFR-negative. FISH in these regions showed low-level amplification of *CCND1*.

Based IHC and FISH analyses, the present tumor consisted exclusively of *CCND1*-amplified cells; of these, 60% exhibited *ERBB2* amplification and ERBB2 overexpression, 30% exhibited *EGFR* amplification and EGFR overexprssion, and 10% lacked any *ERBB2* or *EGFR* amplification or overexpression.

Discussion

The reported frequencies of gene amplification for *EGFR* and *ERBB2* in esophageal SCC are 15% [3] and less than 5%,[4, 5] respectively, and a large comprehensive study of advanced gastric cancers based on single nucleotide polymorphism shows that co-amplification of two or more RTK genes in the same tumor fundamentally does not occur.[9, 13] Our dual-technique study involving MLPA and FISH analysis of advanced gastric cancers also supports this conclusion.[9] Therefore, the co-amplification of *EGFR*

and *ERBB2* found in the present case is apparently an exceptionally rare phenomenon. However, in these previous studies, [9,12] it is possible that small fractions of amplified cells evaded detection due to a dilution effect inherent to studies that involve DNA extracted from large tumors. Actually, contrary to these reports of RTK exclusivity, our previous dual-technique study involving FISH and IHC using whole-tissue sections from gastric cancers showed indications of co-amplification of *EGFR* and *ERBB2*; however, these data were not statistically significant.[14] In our more recent study focusing on the co-amplification of *ERBB2* and other RTK genes, we found that of 51 tumors with prominent *ERBB2* amplification, 14% (7/51) of the tumors displayed some *EGFR* co-amplification.[15] And intriguingly, even in these tumors, the gene co-amplification did not occur within individual cells.

In the present case, three genetically different clones (*ERBB2* amplified, *EGFR* amplified, and both–negative) had *CCND1* amplification in common. These findings suggested that the amplification of either *ERBB2* or *EGFR* occurred after *CCND1* amplification. Because ERBB2 amplification was evident in the carcinoma *in situ*, it is most likely that the *ERBB2* amplification occurred before invasion. Probably one of the cellular functions induced by the action of cyclin D1 is destabilization of the cellular genome,[16] and such destabilization could facilitate subsequent *ERBB2* or *EGFR*

amplification. This hypothesis may be supported by findings from our previous studies in which mucosal cancers that were homogeneously positive for *MYC* alone or *MYC* and *CCND1* has small separated foci with *ERBB2* or *EGFR* amplification.[15, 17] During cancer progression, a single prominent clone with a single RTK gene amplification propagates under Darwinian selection to the exclusion of other less prominent clones; consequently, advanced cancers do not exhibit co-amplification of RTK genes. However, predominance of either of the two clones was apparently not decisive in this chimeric SCC; alternatively, pre-operative chemotherapy may have effectively selected against the poorly differentiated EGFR-positive clone, which was less differentiated and more infiltrative, that might have outcompeted/excluded the *ERBB2*-amplified clone in the absence of the chemotherapy.

Notably, amplification of RTK genes is not highly characteristic of esophageal SCC carcinogenesis. No clear association is evident between RTK amplification and clinicopathological parameters: there is no significant difference in the incidence of *EGFR* amplification between Tis/T1 vs T2-4 [3], and *ERBB2* amplification is unrelated to pT, pN, pM, grade, or UICC-status [5]. Nevertheless, this *EGFR-ERBB2* co-amplification may be clinically significant because the overwhelming number of studies on cancers of the breast, colon, or stomach demonstrate that these particular RTKs when

overexpressed because of gene amplification are promising candidates for molecular-targeted therapies.

Fortunately, in the present case, because of the chemotherapy-surgery combination, the cancer was resectable, and no recurrence has been found. However, if the cancer recurs, one therapeutic option may be molecular-targeted therapy. In this case, theoretically lapatinib, a dual tyrosine-kinase inhibitor with activity against EGFR and ERBB2, could be used first. There is evidence that increased cyclin D expression provides resistance to therapies directed at EGFR.[18] In head and neck SCC cell lines, overexpression and /or amplification of *CCND1* is associated with decreased efficacy of the selective EGFR tyrosine-kinase inhibitor gefitinib. [19] Although it is in the early stage of clinical trials for lung cancer, a cyclin D1-repressing rexanoid bexarotene enhances the effect of the EGFR inhibitor erlotinib. Therefore, it is possible that co-amplification of *CCND1* and *EGFR* could be an important target of this combination therapy. [20]

In conclusion, the relatively rare oncogenic change found this case gave us an important insight into the clonal evolution of esophageal SCC and also provided us useful information with which to plan personalized molecular-targeted therapy.

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Disclosure Statement

The authors declare that no conflict of interest exist with regard to this study.

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Figure legends

Figure 1.

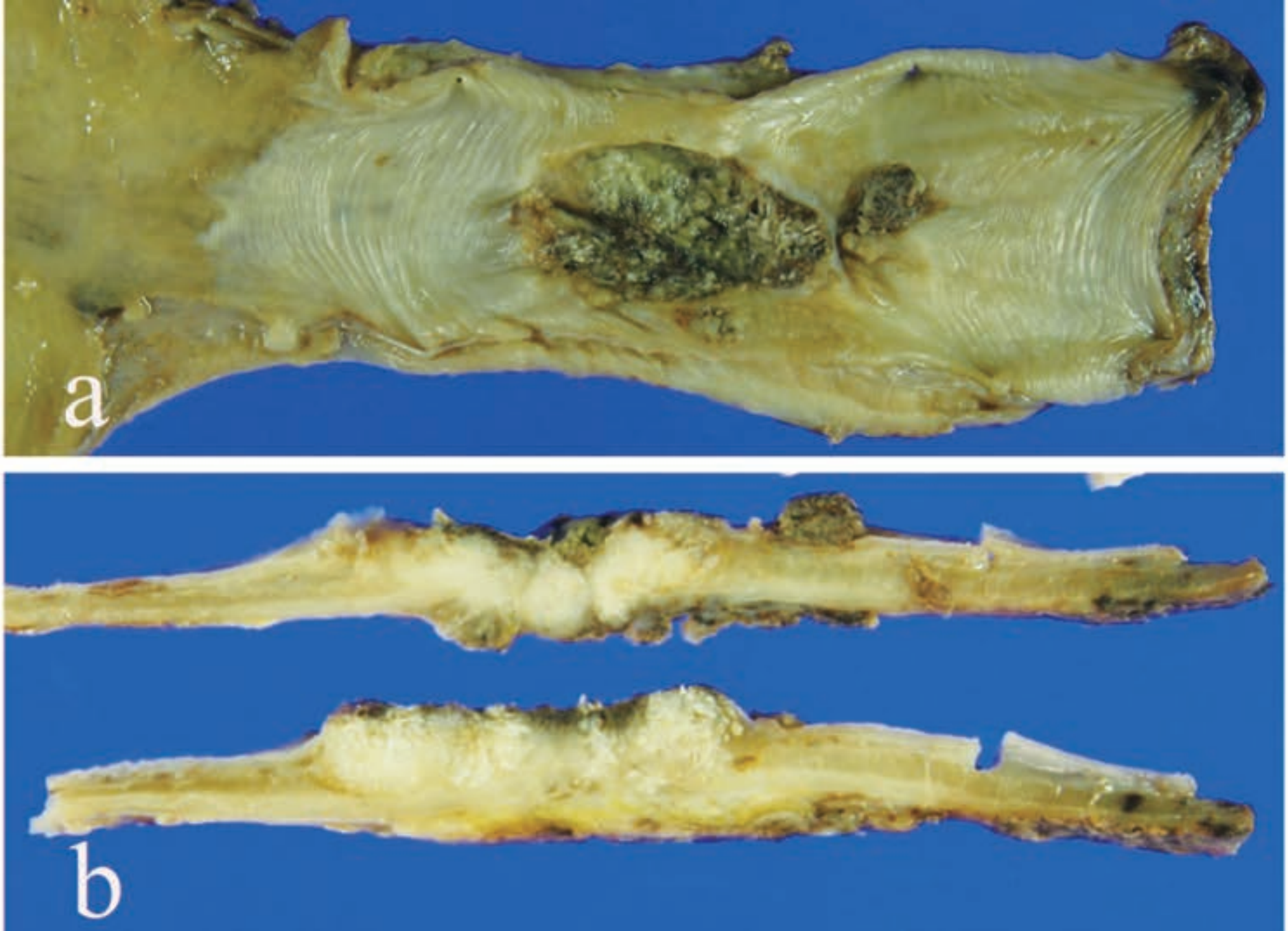
Resected esophagus. An ulcerative and infiltrative tumor from the thoracic esophagus (a). Cut surfaces show the tumor invaded the adventitia (b).

Figure 2

Results from immunohistochemistry (IHC) and a figure showing chemotherapeutic effects. Well-differentiated squamous cell carcinoma (SCC) was positive for ERBB2 (a); the poorly differentiated SCC was EGFR-positive, or both ERBB2- and EGFR-negative (b). Necrotic cancer nests with foreign body reaction were found (c). Mucosal cancer was also positive for ERBB2 (d). Cyclin D1-positive nuclei were scattered evenly throughout the tumor (e).

Figure 3

Results of fluorescence *in situ* hybridization (FISH). The ERBB2-positive cells and the EGFR-positive cells had clustered signals of *ERBB2* (a: orange signals, *ERBB2*; green signals, centromere 17) or *EGFR* (b: orange signals, *EGFR*; green signals, centromere 7), respectively. Based on dual-color FISH images, no single individual cell had both types of amplification (c: green signals, *ERBB2*; orange signals, *EGFR*). Most cancer cells showed increased *CCND1* copy number relative to centromere 11 copy number. (d: orange signals, *CCND1*; green signals, centromere 11). Dual-color FISH demonstrated that *ERBB*-2-amplified cells and *EGFR*-amplified cells each had co-amplification of *CCND1* (e: green signals *ERBB2*; orange signals, *CCND1*. f: green signals, *EGFR*; orange signals, *CCND1*).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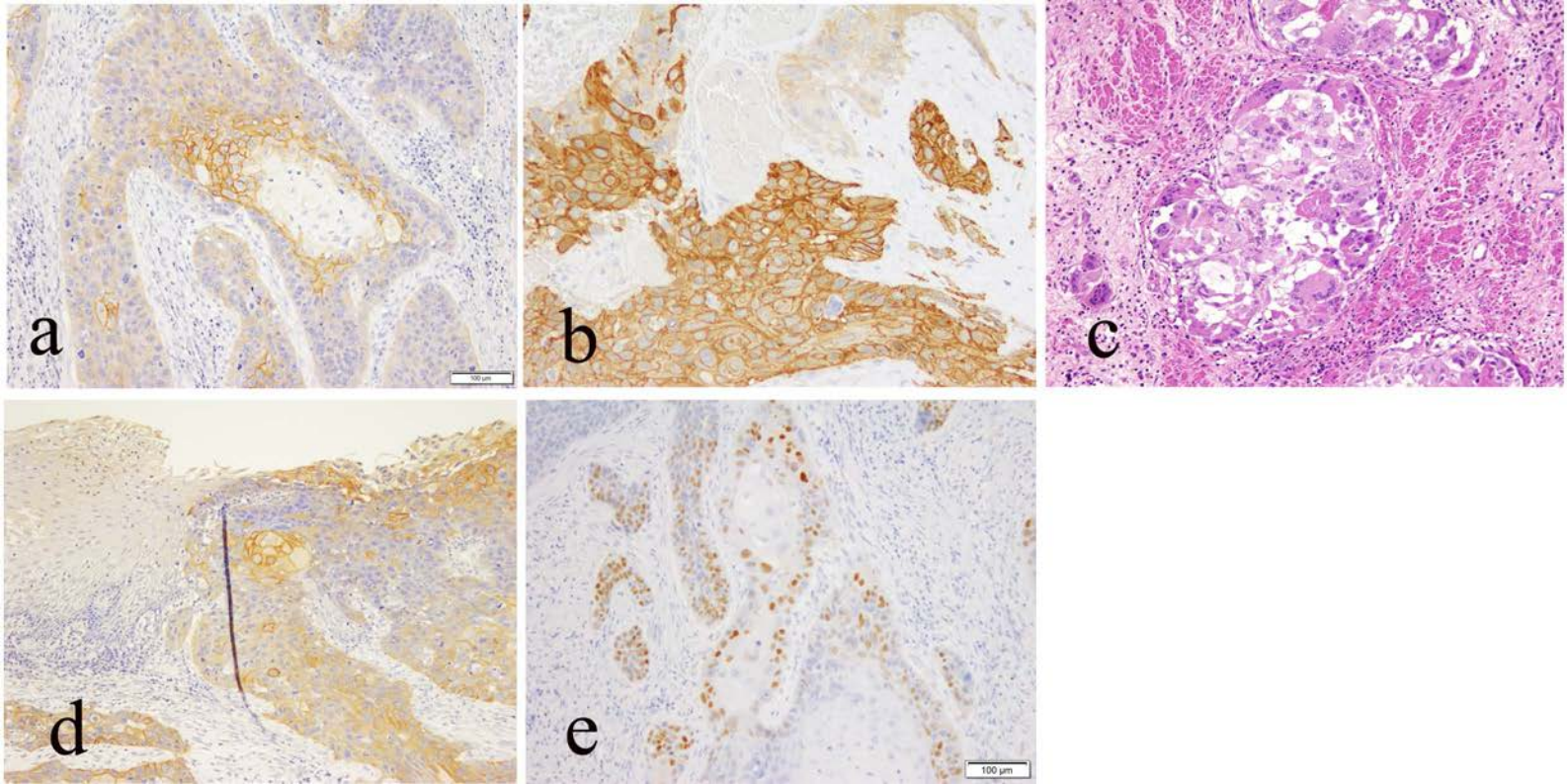
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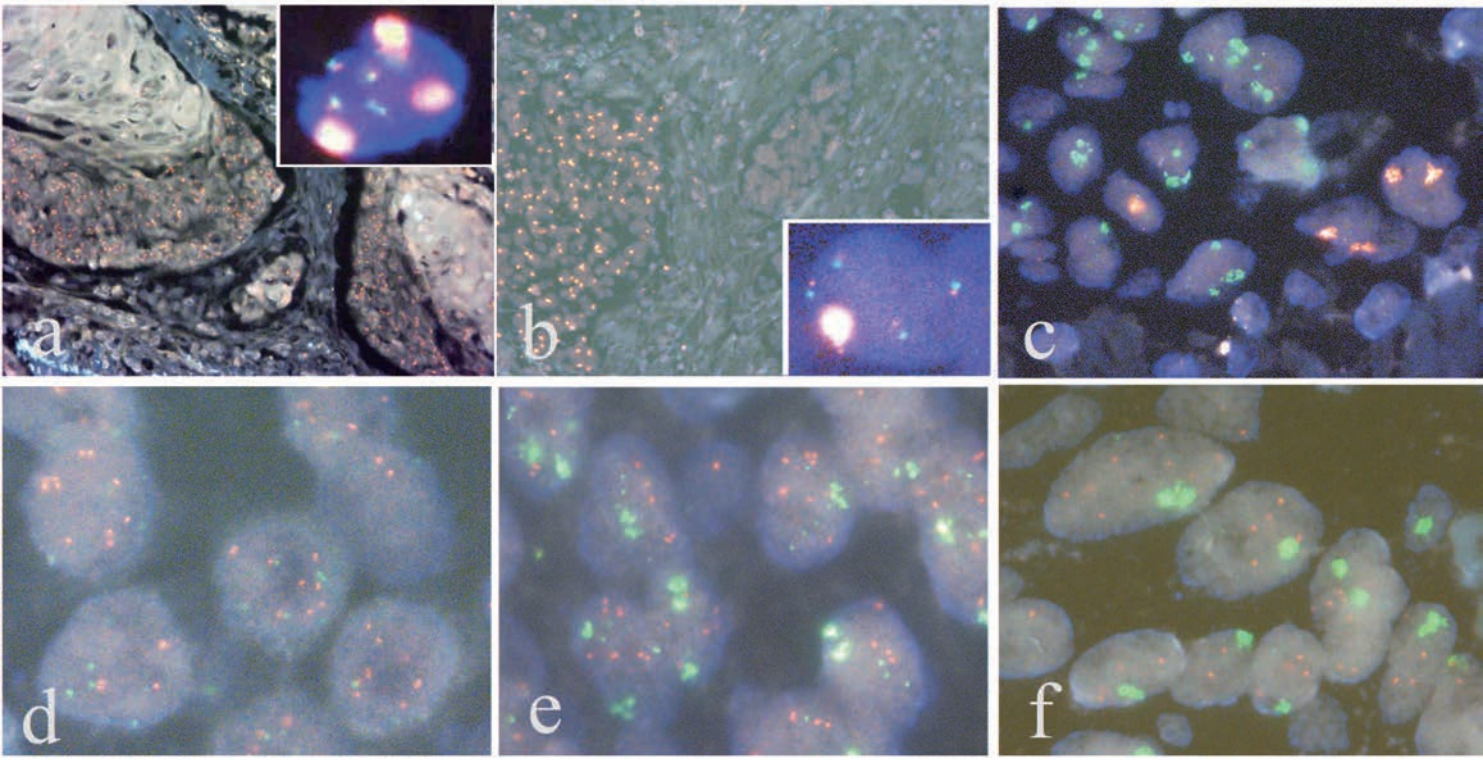
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Table 1 MLPA peak values

Name of gene (chromosomal locus)	EGFR (7p11.2)		CCND1(11q13.3)			ERBB2 (17q12)	
Probe location	Exon 8	Exon 22	Exon 2	Exon3	Exon 5	Exon 7	Exon 29
	3.23	3.14	1.45	1.54	1.37	5.62	9.51