Intratumoral heterogeneous amplification of *ERBB2* and subclonal genetic diversity in gastric cancers revealed by multiple ligation-dependent probe amplification and fluorescence *in situ* hybridization

Running title: Gene amplification of *ERBB2* in gastric cancers

Ryosuke Tajiri MD[1], Akishi Ooi MD[1, 2], Takashi Fujimura MD[3], Yoh Dobashi MD[4], Takeru Oyama MD[1], Ritsuko Nakamura MD[1], Hiroko Ikeda MD[2]

[1]Department of Molecular and Cellular Pathology, Graduate School of Medical Science, Kanazawa University, Ishikawa 920-8641, Japan
[2]Pathology Section, University Hospital, Kanazawa University, Ishikawa 920-8641, Japan
[3]Department of Surgery, Graduate School of Medical Science, Kanazawa University, Ishikawa 920-8641, Japan
[4]Department of Pathology, Saitama Medical Center, Jichi Medical University, Saitama 330-8503, Japan

Correspondence to: Akishi Ooi, Department of Molecular and Cellular Pathology Graduate School of Medical Science, Kanazawa University, Ishikawa 920-8641, Japan.
E-mail: aooi@med.kanazawa-u.ac.jp

No conflicts of interest were declared.

Key words: ERBB2, HER2, gene amplification, gastric cancer, FISH, MLPA

Abstract

A humanized monoclonal antibody against ERBB2 is used in neoadjuvant therapy for gastric cancer patients. A critical factor in determining patient eligibility and predicting outcomes of this therapy is the intratumoral heterogeneity of *ERBB2* amplification in gastric adenocarcinomas. The aims of this study are to assess the underlying mechanisms of intratumoral heterogeneity of *ERBB2* amplification and to characterize the diversity of co-amplified oncogenes, such as *EGFR, FGFR2, MET, MYC*, *CCND1* and *MDM2*, and to examine the usefulness of multiple ligation-dependent probe amplification (MLPA) in the semi-comprehensive detection of these gene amplifications. A combined analysis of immunohistochemistry and fluorescence *in situ* hybridization (FISH) revealed *ERBB2*-amplified cancer cells in 51 of 475 formalin-fixed paraffin-embedded gastric adenocarcinomas. The fraction of amplification-positive cells in each tumor ranged from less than 10% to almost 100%. Intratumoral heterogeneity of *ERBB2* amplification, defined as less than 50% of cancer cells positive for *ERBB2* amplification, was found in 41% (21 of 51) of ERBB2-amplified tumors. The combined analysis of MLPA and FISH revealed that *ERBB2* was co-amplified with *EGFR* in seven tumors, *FGFR2* in one tumor, and *FGFR2* and *MET* in one tumor; however, the respective genes were amplified in mutually exclusive cells. Co-amplified *ERBB2* and *MYC* coexisted within single nuclei in four tumors, and one of these cases had suspected co-amplification in the same amplicon of *ERBB2* with *MYC*. In conclusion, the amplification status of *ERBB2* and other genes can be obtained semi-comprehensively by MLPA and could be useful to plan individualized molecularly targeted therapy against gastric cancers.

Introduction

Gastric cancer is the fourth most common human malignancy and the second most common cause of cancer-related death worldwide (1). The clinical outcome of gastric cancer has gradually improved; however, the prognosis of patients with advanced disease is still dismal. Gastric carcinomas in the initial stages, such as early gastric carcinomas as defined by the Japanese Gastric Cancer Research Society (2), are carcinomas that are confined to the mucosa and the submucosa that are usually endoscopically or surgically resectable and can be cured (3). However, the majority of patients with advanced gastric carcinomas, defined as carcinomas that penetrate the muscle layer or beyond (2), have metastatic lesions and/or inoperable carcinomas and have a poor prognosis (4). Thus, novel therapeutic modalities are urgently needed for the treatment of late-stage gastric carcinomas. One potentially useful approach is the molecular targeting of ERBB2 (5, 6).

ERBB2 (also called HER2) is a 185-kDa transmembrane tyrosine kinase receptor. Overexpression as a result of *ERBB2* gene amplification on chromosome 17q11.2-q12 has been observed in solid tumors including gastric and breast cancers (7). *ERBB2* is amplified in 7%-27% of gastric cancers (8-11). In breast cancers, overexpressed ERBB2 is a therapeutic target of trastuzumab, a humanized monoclonal antibody that binds to the extracellular juxtamembrane domain of ERBB2 and inhibits the proliferation and survival of ERBB2-overexpressing cancer cells (12). In 2009, an open-label, international phase 3, randomized controlled trial (ToGA, Trastuzumab for Gastric Cancer) found that the addition of trastuzumab to cisplatin-based chemotherapy significantly improved the overall survival of advanced gastric cancer patients with over-expressed and/or amplified *ERBB2* as compared to chemotherapy alone (13). As the target of therapy with trastuzumab, the evident difference between gastric cancer and breast cancer is the intratumoral heterogeneity of *ERBB2* amplification (7).

It is noteworthy that recent comprehensive genomic analyses of copy number alterations with a high-resolution single nucleotide polymorphism array (14) and oligonucleotide array comparative genomic hybridization (aCGH) (15) revealed that genes encoding receptor tyrosine kinases (RTKs), such as *ERBB2*, *FGFR2*, *MET* and *EGFR*, in addition to *MYC*, *CCND1* and *MDM2*, were frequently amplified in gastric cancers. It is most likely that

intratumoral heterogeneity of *ERBB2* amplification is based on common chromosomal instability where new amplification of some of these genes begets new cancer cells in which *ERBB2* amplification is dispensable, or co-amplification of new genes with *ERBB2* gives sister cancer cells additional or synergistic growth advantages(16).

In tumors with genetic heterogeneity, the genomic landscape, as determined by results from single gene aberration assays, is critical for personalized-medicine strategies. For example, when molecularly targeted therapy against ERBB2 is used, ERBB2-negative cancer cells, if any, may survive or exhibit overgrowth after ERBB2-positive cancer cells have been killed, as we have previously reported in a case of breast cancer (17). Thus, the semi-comprehensive analysis of therapy-related genes is mandatory. Multiplex ligation-dependent probe amplification (MLPA) is a new, high-resolution method for detecting numerous copy number variations in genomic sequences in a single reaction requiring only small amounts of DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues (18, 19). A detection kit for *ERBB2, EGFR, MET, MYC, CCND1* and *MDM2* and a detection kit for *FGFR2*, which we utilized in the present study, have recently become commercially available.

In the present study, we sought to identify the heterogeneity of *ERBB2* amplification in gastric cancers and to characterize the possible diversity of amplified oncogenes with or without *ERBB2* amplification. To achieve these goals, we tested the reliability of the recently developed MLPA as a tool to semi-comprehensively detect molecular changes.

Materials and Methods

Patients

A total of 475 patients with gastric adenocarcinoma (310 early and 165 advanced tumors) who underwent surgery or endoscopic resection at the Department of Surgery in Kanazawa University Hospital between 2005 and 2010 were examined. Cancer staging was performed according to the TNM cancer staging system of the American Joint Committee of Cancer (20). The World Health Organization Classification of Tumors (21) was used to

determine histological classification, i.e., as differentiated (tubular adenocarcinoma, papillary adenocarcinoma) or undifferentiated adenocarcinoma (mucinous adenocarcinoma, poorly cohesive carcinomas, mixed carcinoma, carcinoma with lymphoid stroma and unclassified carcinoma). This laboratory study was approved by the Institutional Review Board at Kanazawa University Hospital (Approval No 181), and written informed consent was obtained from all patients. Serial sections cut from representative FFPE cancer tissues were used for hematoxylin-eosin staining, immunohistochemistry (IHC), MLPA and FISH.

IHC

IHC for ERBB2 and EGFR proteins was performed on all primary tumors. A polyclonal antibody against the internal domain of the human ERBB2 protein (Nichirei, Tokyo, Japan; working dilution, 1:400) and a monoclonal antibody against the external domain of human EGFR (Novocastra Lab, Newcastle, UK; working dilution, 1:20) were used. Antibody binding was visualized by the LSAB™ system (Dako, Glostrup, Denmark). For evaluation of the IHC staining, each tumor, or portion of tumor, was scored by using a four-tier system (0, 1+, 2+, 3+) according to the criteria recommended by Dako for the HercepTest™, except that the quantity of positive cells was not considered.

FISH

Areas with positive IHC staining (2+ and 3+) for ERBB2 and EGFR and areas with amplification and gain of *FGFR2*, *MET*, *MYC*, *CCND1*, *CDK4*, or *MDM2*, as determined by MLPA, were examined for gene amplification by FISH. When the gene amplification was positive in the primary tumors, their nodal metastases then underwent FISH examination for amplification of the genes.

For FISH probes, bacterial artificial chromosomes RP11-62N23, RP11-339F13, RP11-62L18, RP11-75I20, RP11-440N18, RP11-775J10, RP11-300I6, and RP11-571M6, specific to *ERBB2*, *EGFR*, *FGFR-2*, *MET*, *MYC*, *CCND1*, *CDK4* and *MDM2*, respectively, were acquired from BACPAC Resources (Oakland, CA) and labeled with SpectrumOrange™ or SpectrumGreen™ with a nick translation kit (Abbott Laboratories, Abbott Park, IL). For the detection of gene amplification, a SpectrumGreen™-labeled pericentromeric probe (Abbott), specific to each chromosome on which the

particular gene was located, was co-hybridized to standardize the chromosome number. The tumors exhibiting co-amplifications of different genes were further examined by simultaneous hybridization with two probes specific to the genes labeled by different fluorescent markers to determine the co-existence of the amplified genes in single cells and single amplicons.

The removal of protein from the tissue sections was conducted as previously described (10). Denaturation, hybridization, and post-hybridization washing were performed according to Abbott's protocol. The tissue sections were counterstained with DAPI II (Abbott) and examined with a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Triple Bandpass Filter set (Abbott) for DAPI II, SpectrumOrange^{TM} and SpectrumGreen^{TM}, and a filter set specific to SpectrumOrange^{TM} or SpectrumGreen^{TM}. The FISH images were recorded through a cooled charge-coupled device camera (DP-70) linked to a computer software program (DP Manager, Olympus).

FISH results were scored manually, and gene amplification was determined according to the criteria approved by the American Society of Clinical Oncology/College of American Pathologists for *ERBB2* amplification, which is more than six gene copies per nucleus or gene signals/centromere signals > 2.2 (22). The fraction (%) of the *ERBB2*-amplified tumor cells and the sizes of the tumor areas occupied by the *ERBB2*-amplified tumor cells were determined in the representative sections by using IHC and FISH. The tumors with less than 50% of *ERBB2*-amplified cells were arbitrarily defined as tumors with heterogeneous amplification of *ERBB2*.

MLPA

To efficiently identify co-amplified genes and genes that were amplified exclusively with *ERBB2*, MLPA was performed separately, if possible, in the *ERBB2*-amplified tumor areas and the non-amplified areas. Combined with IHC and FISH, the *ERBB2*-amplified tumor areas and the non-amplified areas, if any, were identified in the FFPE tissues on glass slides. By using adjacent 6-μm-thick sections, DNA was extracted manually from each section according to the manufacturer's protocol. Amplified areas that were as large as possible were selected to obtain ample DNA, but at the same time the *ERBB2*-non-amplified cancer cells and non-neoplastic cells were excluded as much as possible. The *ERBB2*-non-amplified areas were selected to exclude amplified cells as much as possible. As a result, more than 1-μg DNA samples

each with OD_{260}:OD_{280} ratios within 1.1-1.7 were obtained from *ERBB2*-amplified areas in all 51 tumors, and in 20 of these tumors samples were obtained from both the amplification-positive and amplification-negative areas.

Forty gastric cancers without *ERBB2* amplification were selected at random from 314 differentiated-type gastric adenocarcinomas for DNA examination. DNA from cell lines MKN7, A431, KATOIII, HSC39, and MKN45, which had previously been confirmed to have amplified *ERBB2, EGFR, FGFR2, FGFR2* and *MYC*, and *MET*, respectively, were used as positive controls (23, 24).

MLPA analysis was performed by using two kits from MRC-Holland (Amsterdam, The Netherlands). The SALSA MLPA KIT P175-A2 Tumour-Gain kit contains probes for 24 genes including *ERBB2, EGFR, MET, MYC, CCND1* and *MDM2*, and the SALSA MLPA probemix P231-A2 FGF10-FGFR2 contains probes for *FGFR2* and *FGF10*. The MLPA PCR products were separated on an ABI-310 capillary sequencer (Applied Biosystems, Foster City, CA) and interpreted with Genemapper software (Applied Biosystems). Data analysis was performed with Coffalyser MLPA-DAT software (version 9.4, MRC-Holland) to generate normalized peak values. Peak values below 0.7 were defined as lost, between 0.7 and 1.3 as normal, between 1.3 and 2.0 as gain, and >2.0 as amplified, as previously established (19).

Results

Amplification of *ERBB2*

Among a total of 475 tumors, ERBB2 overexpression by IHC was found in 60 tumors. By FISH, 51 of 60 ERBB2-overexpressing tumors (8 tumors with 2+ overexpression and 43 tumors with 3+ overexpression) had gene amplification of *ERBB2*; most of them appeared as tightly clustered or numerous scattered signals, suggesting amplicons in homogeneously staining regions (HSR) or double minute chromosomes (DM) (25). *ERBB2*

amplification was significantly more frequent in differentiated carcinomas than in undifferentiated carcinomas (p=0.0078). Among the 51 tumors, 23 were advanced gastric cancers. No significant correlation was found between the existence or extent of *ERBB2* amplification and the location, T stage or N stage of the tumor. The amount of amplification-positive cells in each tumor ranged from almost all cancer cells (Fig. 1A), to small distinct clusters (Fig. 1B, arrow), or confluent tubules (Fig. 1C&D), which accounted for less than 10% of the total cancer cells. Otherwise, the amplified and non-amplified tumor cells were mosaic admixed (Fig. 1E&F). Fractions of the *ERBB2*-amplified cells and sizes of areas occupied by the amplified cells in each tumor are shown in Table 1. Intratumoral heterogeneity of *ERBB2* amplification was found in 21 (41%) of the 51 tumors. The numbers of tumors with different fractions of *ERBB2*-amplified cells are summarized separately in advanced and early gastric cancers in Figure 2.

MLPA analysis of *ERBB2* amplification

The MLPA analysis was successfully performed on all of the FFPE samples. The mean MLPA peak values of the 51 tumors with FISH-proven *ERBB2*-amplification are shown in Table 1. Among the 51 tumors, amplification and gain of *ERBB2* by MLPA were found in 32 tumors (63%) and 14 tumors (27%), respectively, and five tumors had peak values of *ERBB2* within the normal range (Table 1). MLPA could detect small *ERBB2*-amplified areas (fraction of approximately 35%), such as in case 47 (Figs. 3A, B and D). Cases with less than 10% of *ERBB2*-amplified cells (for example, case 13) could be identified as amplification if samples containing at least approximately 30% of *ERBB2*-amplified cells could be prepared by trimming. However, in the five tumors with normal MLPA values, *ERBB2*-amplified cells were confined to minute areas; thus, it was technically difficult to increase the fraction of *ERBB2*-amplified tumor cells.

In 20 tumors, MLPA samples could be obtained separately from areas with positive and negative amplification of *ERBB2* (Table 1). The 20 *ERBB2*-negative areas of the *ERBB2*-positive cancers showed neither

amplification nor gain of *ERBB2*. The MLPA results of *ERBB2* and numbers of tumors with different fractions of *ERBB2*-amplified cells are shown separately in advanced and early gastric cancers in Figure 2.

Amplification of genes other than *ERBB2*

The results of amplification of genes other than *ERBB2* are summarized in Tables 1 and 2, and representative cases are shown in Figures 3 and 4.

EGFR

The two tumors categorized as having *EGFR* amplification by MLPA also proved to have *EGFR* amplification by FISH. Among the five tumors categorized as gain, three tumors showed *EGFR*-amplified cancer cells (Fig. 3C and E). By performing FISH after IHC we detected two additional tumors with small areas of *EGFR* amplification. In all seven *EGFR*-amplified tumors, *ERBB2*- and *EGFR*-amplified cell populations were found in the vicinity, but they were separated and mutually exclusive, as shown in Figs. 3A-E.

FGFR2 & MET

MLPA successfully detected amplifications of *FGFR2* in two cases; in addition, the gain of *MET* in one of them led to detection of the amplification of this gene by FISH. This tumor consisted of *ERBB2*-positive tubular adenocarcinoma and *ERBB2*-negative poorly differentiated adenocarcinoma, and the latter had scattered *MET*- and *FGFR2*-amplified cells with mutually exclusive distributions, in addition to both negative cells as shown by dual-color FISH (Figs. 4A-C).

MYC

By MLPA analysis, amplification and gain of *MYC* were found in four and eight tumors, respectively. Gene amplification of *MYC* was confirmed by FISH in all four tumors with amplification and in three tumors with gain. In the remaining five tumors with gain, tumor cells were found to have a few additional copy numbers of *MYC* genes to the number of centromere 8. However, their ratios were less than 2.2, and the average copy numbers of *MYC* were less than 7; thus, they were not included in the amplified tumors (Suppl Fig. 1).

Coexistence of amplified *MYC* and *ERBB2* within single nuclei was found in

four tumors, and *MYC/FGFR2* and *MYC/EGFR* in a case respectively. In a remaining tumor, amplification of *ERBB2* and *MYC* was found in different cells. In one of the tumors with co-amplification of *MYC* and *ERBB2* (case 21) and another tumor with co-amplification of *MYC* and *FGFR2* (case 11), the co-amplified signals were exclusively overlapped and appeared as numerous scattered signals or tightly clustered signals, suggesting that the co-amplified genes were located on the same amplicons in DM and HSR, respectively (25).

CCND1

MLPA showed three tumors with amplification of *CCND1*, and amplification was confirmed in the three cases by FISH (Table 2). Co-amplification of *CCND1* and *ERBB2*, *EGFR*, and *MYC* were found in three tumors; however, no discernible correlation between the distributions of *CCND1* and the co-amplified genes was found in these tumors.

Other genes

Amplification and gain of *TOP2A* was found in eight and 15 tumors, respectively. Gain of *MDM2* was found in two tumors; however, they showed no amplification by FISH. The other 17 genes detectable by the two MLPA kits showed no amplification or gain.

ERBB2–non-amplified tumors

All of the 40 *ERBB2*-negative control tumors determined by IHC showed normal peak values of *ERBB2*. Thus, the specificity of the MLPA was 100%. Among genes with amplification or gain, as shown in Table 2, there were amplifications of *FGFR2* in three tumors and amplification of *MYC*, *CDK4* (Suppl Fig. 2) and *MDM2* (Suppl Fig. 3) in one tumor each by FISH.

Lymph node status

Amplified genes in the metastatic cancer cells examined by FISH are shown in Table 1.

Discussion

In the present study, the combined analysis of IHC and FISH revealed that

51 (11%) of 475 of gastric cancers contained *ERBB2*-amplified cells ranging from less than 10% to almost 100% in each tumor. At the present time, there is neither a definition nor a general consensus of intratumoral heterogeneity of *ERBB2*-amplification in gastric cancers. In regard to breast cancers, the College of American Pathologists expert panel has adopted 30% as the cutoff for homogeneous ERBB2 overexpression and recommended that cancers containing between 5% and 50% of *ERBB2*-amplified cells, as determined by FISH, should be reported as heterogeneous for *ERBB2* gene amplification (22).

In the present study, which used this 50% cutoff value, intratumoral heterogeneity of *ERBB2* amplification was found in 41% (21 of 51) of *ERBB2*-amplified tumors; conversely, homogenous *ERBB2*-amplification was identified in 59% of *ERBB2*-amplified tumors. Because molecular target-based therapy with trastuzumab seems to be promising for advanced gastric cancers, if we seek cancers with homogeneous *ERBB2*-amplification in advanced gastric cancers, 9.7% (16 of 165 tumors) would meet this criterion.

Although the primary aim of the present study was not to evaluate the resolving power of MLPA, MLPA could successfully identify samples containing as little as 30% positive cells as having gene amplification. Thus, if MLPA had been performed with whole cancer sections from the 16 advanced gastric cancers with more than 50% amplified cancer cells, the MLPA analyses could have identified all of the cases except one as amplification (Fig. 2). A new research project may be possible that aims at determining whether MLPA can be a really useful clinical test to select trastuzumab-eligible advanced gastric cancers.

Recently, genes encoding RTKs have attracted much attention as targets of molecular therapy. An aCGH study reported that *ERBB2, FGFR2, EGFR, MET* and *KRAS* were fundamentally not co-amplified and estimated that approximately 40% of gastric cancer patients may be treatable with RTK/RAS-directed therapies (14). Similarly, another FISH study examining 489 esophagogastric adenocarcinomas described that the amplification of *ERBB2, MET,* and *EGFR* were, with one exception (*MET* and *EGFR* positive), mutually exclusive events on a tumor-by-tumor basis; thus, approximately 16% of gastric cancer patients are potentially suitable for targeted treatment (26). However, co-amplification of RTK genes in single tumors may not be infrequent because in the present study, which was limited to the co-amplifications of *ERBB2* and other RTK genes, among 51 *ERBB2*-amplified

tumors co-amplification with *EGFR* occurred in 14% (7/51) of tumors, with *FGFR2* in one tumor, and with *MET* and *FGFR2* in one tumor. Theoretically, for the tumors having co-amplification of *ERBB2* and other RTK gene(s) mutually exclusively on a cell-by-cell basis, cocktails of trastuzumab and other monoclonal antibodies, or trastuzumab and other small molecule inhibitors could be candidate treatments. When *ERBB2* and *EGFR* are co-amplified, a dual inhibitor of both ERBB2 and EGFR, e.g., lapatinib, may be effective (27).

The MLPA analysis may provide further useful guidance in the selection of appropriate molecular targets in gastric cancer. From preliminary analyses from a clinical study of breast cancer, the NSABP B-31 trial, it was suggested that the tumors with co-amplified *ERBB2* and *MYC* have a remarkably favorable prognosis with adjuvant trastuzumab treatment (28). In an early experimental study, small molecule inhibitors of FGFR2 induced apoptosis in SNU 16, which has co-amplification of *FGFR2* and *MYC*, but induced only growth arrest in KATOIII, which has only *FGFR2* amplification (29). Thus, we may expect additional effects from the co-amplification of *MYC* in the targeted therapy against *FGFR2*-amplified cancer cells. Furthermore, information of the amplification of *CCND1* is useful because expression of CCND1 may cause resistance to cisplatin, which was co-administered with trastuzumab in the ToGA clinical trial.

Amplification and gain of *TOP2A* were found in eight *ERBB2*-amplified cancers. *TOP2A* amplification was exclusively observed with the concomitant amplification of *ERBB2* (9). *TOP2A* encodes an enzyme required for DNA replication, topoisomerase IIα, and clinical studies have confirmed that breast cancer patients with *TOP2A* gene amplification are more sensitive to topoisomerase IIα-based therapy (30). As *TOP2A* resides very close to *ERBB2*, being separated only by approximately 680 kb (UCSC Genome Browser on Human Feb 2009 Assembly), MLPA may have higher resolving power than FISH to determine if the amplicon containing *ERBB2* extends to the locus of *TOP2A*. In the present study, two additional candidates for molecular targeting, CDK4 and MDM2, were found in *ERBB2*-non-amplified gastric cancers.

In conclusion, MLPA is a feasible and easy-to-use tool to collect semi-comprehensive information on *ERBB2* and possible target genes, and MLPA can be useful in planning individualized molecularly targeted therapy against gastric cancers.

Acknowledgements

This work was financially supported by the Japanese Ministry of Education, Sports, Science and Culture [Nos. C25460452 (AO), C23590409 (YD) and Young Scientists (B) (25860266) (TO)] and the Smoking Research Foundation (YD).

No conflicts of interests were declared.

Figure legends

Figure 1

Representative cases with different percentages of cells with overexpression and amplification of *ERBB2*. (A, B, C and E, IHC for ERBB2). Arrow indicates the small cluster of ERBB2-overexpressing cells (panel B). The region with the black rectangle in panel C corresponds to the field in panel D, and panel F is a serial section of panel E. FISH demonstrates that the ERBB2-overexpressing cells have gene amplification of *ERBB2* (D and F: orange signals, *ERBB2*; green signals, centromere 17). (A, case 21; B, case 11; C and D, case 18; E and F, case 44)

Figure 2

Numbers of cases with *ERBB2* amplification (black bars), *ERBB2* gain (gray bars) and normal *ERBB2* (white bars) as determined by MLPA in different fractions of *ERBB2*-amplified cells detected by FISH.

Figure 3

Representative images of co-amplification of *ERBB2*, *EGFR*, *MYC*, *CCND1*, and *FGFR2*. Intramucosal adenocarcinoma with heterogeneous *ERBB2* amplification (A-F, case 47). The circled region with dotted line in panel A was used for MLPA analysis. (MLPA peak values: *ERBB2*, 6.36; *EGFR*, 1.32, *MYC*, 3.8; *CCND1*, 3.00) The regions with the black rectangles in panel A correspond to the fields in panels B and C. IHC shows that approximately 35% of cancer cells in the circled region with dotted line in panel A was ERBB2-overexpressing cancer cells (B) and approximately 6% EGFR-overexpressing cancer cells (C). The regions labeled with d, e, and f in

panel B and panel C correspond to the fields in panel D, E, and F respectively. Dual-color FISH shows co-amplifications of *ERBB2* and *MYC* (D: orange signals, *ERBB2*; green signals, *MYC*), and *EGFR* and *MYC* (E: orange signals, *EGFR*; green signals, *MYC*). Almost all of the cancer cells have co-amplification of *MYC* (orange signals) and *CCND1* (green signals) (F) Co-amplification of *ERBB2* and *MYC* (G-I) (case 21: MLPA peak values: *ERBB2*, 21.9; *MYC*, 15.4), and *FGFR2* and *MYC* (J-L) (case 11:MLPA peak values: *FGFR2*, 62.3; *MYC*, 15.1) Dual-color FISH shows the signals of *ERBB2* (orange) and *MYC* (green) in case 21, and the signals of *FGFR2* (green) and *MYC* (orange) in case 11 are exclusively overlapped. (G and L, triple-band filter; H and K, SpectrumOrange™-specific filter: I and L, SpectrumGreen™-specific filter).

Figure 4

Advanced gastric adenocarcinoma with co-amplification of *ERBB2*, *MET*, and *FGFR2* (case 4: MLPA peak values: *ERBB2*, 3.03; *FGFR2*, 2.83; *MET*, 1.41). Cancer cells in subsets of tubular adenocarcinoma show *ERBB2* amplification (A, dual-color FISH; orange signals, *ERBB2*; green signals, centromere 17) whereas cancer cells of poorly differentiated adenocarcinoma show *MET* amplification or *FGFR2* amplification. (dual-color FISH, B and C; orange signals, *MET*; green signals, *FGFR2*)

Supplemental Figure 1

Mean ratio of *MYC* to CEP8 is 1.9 (case 15).

Supplemental Figure 2

Gene amplification of *CDK4* in an *ERBB2*-negative gastric cancer.

Supplemental Figure 3

Gene amplification of *MDM2* in an *ERBB2*-negative gastric cancer.

References

1. Ferlay J, Parkin DM, Steliarova-Foucher E. Estimates of cancer incidence

and mortality in Europe in 2008. Eur J Cancer 2010; 46, 765-81.
2. Japanese Research Society for Gastric Cancer Committee on Histologic Classification of Gastric Cancer. The General rules for the gastric cancer. Jpn J Surg 1981; 11:140-5.
3. Okabayashi T, Kobayashi M, Nishimori I, et al. Clinicopathological features and medical management of early gastric cancer. Am J Surg 2008; 195, 229-32.
4. Fielding JW, Roginski C, Ellis DJ, et al. Clinicopathological staging of gastric cancer. Br J Surg 1984; 71, 677-80.
5. Okines A, Cunningham D, Chau I. Targeting the human EGFR family in esophagogastric cancer. Nat Rev Clin Oncol; 8, 492-503.
6. Fornaro L, Lucchesi M, Caparello C, et al. Anti-HER agents in gastric cancer: from bench to bedside. Nat Rev Gastroenterol Hepatol; 8, 369-83.
7. Ruschoff J, Hanna W, Bilous M, et al. HER2 testing in gastric cancer: a practical approach. Mod Pathol 2012; 25, 637-50.
8. Okines AF, Cunningham D. Trastuzumab: a novel standard option for patients with HER-2-positive advanced gastric or gastro-oesophageal junction cancer. Therap Adv Gastroenterol 2012; 5, 301-18.
9. Kanta SY, Yamane T, Dobashi Y, Mitsui F, Kono K, Ooi A. Topoisomerase IIalpha gene amplification in gastric carcinomas: correlation with the HER2 gene. An immunohistochemical, immunoblotting, and multicolor fluorescence in situ hybridization study. Hum Pathol 2006; 37, 1333-43.
10. Takehana T, Kunitomo K, Kono K, et al. Status of c-erbB-2 in gastric adenocarcinoma: a comparative study of immunohistochemistry, fluorescence in situ hybridization and enzyme-linked immuno-sorbent assay. Intl J Cancer 2002; 98, 833-7.
11. Yano T, Doi T, Ohtsu A, et al. Comparison of HER2 gene amplification assessed by fluorescence in situ hybridization and HER2 protein expression assessed by immunohistochemistry in gastric cancer. Oncol Rep 2006; 15, 65-71.
12. Hudis CA. Trastuzumab--mechanism of action and use in clinical practice. N Engl J Med 2007; 357, 39-51.
13. Bang YJ, Van Cutsem E, Feyereislova A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet 2010 ; 376, 687-97.
14. Deng N, Goh LK, Wang H, et al. A comprehensive survey of genomic alterations in gastric cancer reveals systematic patterns of molecular exclusivity and

co-occurrence among distinct therapeutic targets. Gut 2012; 61, 673-684.
15. Rossi E, Klersy C, Manca R, Zuffardi O, Solcia E. Correlation between genomic alterations assessed by array comparative genomic hybridization, prognostically informative histologic subtype, stage, and patient survival in gastric cancer. Hum Pathol 2011; 42, 1937-45.
16. Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer 2012; 12, 323-34.
17. Kunitomo K, Inoue S, Ichihara F, et al. A case of metastatic breast cancer with outgrowth of HER2-negative cells after eradication of HER2-positive cells by humanized anti-HER2 monoclonal antibody (trastuzumab) combined with docetaxel. Hum Pathol 2004; 35, 379-81.
18. Bunyan DJ, Eccles DM, Sillibourne J, et al. Dosage analysis of cancer predisposition genes by multiplex ligation-dependent probe amplification. Br J Cancer 2004; 91, 1155-9.
19. Ooi A, Inokuchi M, Harada S, et al. Gene amplification of ESR1 in breast cancers--fact or fiction? A fluorescence in situ hybridization and multiplex ligation-dependent probe amplification study. J Pathol 2012; 227, 8-16.
20. Stomach, In: Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A III eds. AJCC Cancer Staging Manual 7th ed. New york, NY: Springer;2010. p117-20.
21. Fenoglio-Preiser C, Muroz N, Carneiro F et al. Tumours of the stomach. In: Hamilton SR, Aaltonen LA eds. World Health Organization Classification of Tumours. Tumours of the Digestive System. Lyon, France : IARC Press, 2000. p37-66.
22. Wolff AC, Hammond ME, Schwartz JN, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol 2007; 25, 118-45.
23. Takehana T, Kunitomo K, Suzuki S, et al. Expression of epidermal growth factor receptor in gastric carcinomas. Clin Gastroenterol Hepatol 2003; 1, 438-45.
24. Hara T, Ooi A, Kobayashi et al. Amplification of c-myc, K-sam, and c-met in gastric cancers: detection by fluorescence in situ hybridization. Lab Invest 1998; 78, 1143-53.
25. Kobayashi M, Ooi A, Oda Y, Nakanishi I. Protein overexpression and gene amplification of c-erbB-2 in breast carcinomas: a comparative study of immunohistochemistry and fluorescence in situ hybridization of formalin-fixed,

paraffin-embedded tissues. Hum Pathol 2002; 33, 21-8.
26. Lennerz JK, Kwak EL, Ackerman A, et al. MET amplification identifies a small and aggressive subgroup of esophagogastric adenocarcinoma with evidence of responsiveness to crizotinib. J Clin Oncol 2011; 29, 4803-10.
27. Fralick M, Hilton JF, Bouganim N, Clemons M, Amir E. Dual blockade of HER2 - twice as good or twice as toxic? Clin Oncol (R Coll Radiol) 2012; 24, 593-603.
28. Kim C. Bryant J, Horne Z, et al. Trastuzumab sensitivity of breast cancer with co-amplification of HER2 and cMyc in vivo. Breast Cancer Res Treat 2005; 54(Suppl 1), S6 (abstract).
29. Kunii K, Davis L, Gorenstein J, et al. FGFR2-amplified gastric cancer cell lines require FGFR2 and Erbb3 signaling for growth and survival. Cancer Res 2008; 68, 2340-8.
30. Di Leo A, Gancberg D, Larsimont D, et al. HER-2 amplification and topoisomerase IIalpha gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with an anthracycline-based therapy or with cyclophosphamide, methotrexate, and 5-fluorouracil. Clin Cancer Res 2002; 8, 1107-16.

Table 1. Results of MLPA analysis of 51 cases with *ERBB2* amplification

Case No	Depth of invasion	*ERBB2*-positive area				*ERBB2*-negative area		Lymph nodes	
		MLPA values	Fraction (%)	area size (mm)	FISH-detected co-amplified genes	MLPA values	FISH-detected co-amplified genes	M	Amplified genes
1	ss	5.24 [a]	80	5x3		0.95 [c]	*EGFR*		
2	sm	4.65 [a]	80	13x3		1.25 [c]			
3	sm	3.39 [a]	80	10x3		0.95 [c]	*MYC*		
4	se	3.03 [a]	50	6x2		0.98 [c]	*FGFR2, MET*	+	*ERBB2/MET*
5	ss	6.08 [a]	40	3x1	*EGFR*	1.16 [c]		+	*ERBB2*
6	sm	3.13 [a]	30	5x2		1.05 [c]			
7	sm	2.85 [a]	30	9x1		0.93 [c]			
8	sm	2.56 [a]	30	6x1		1.07 [c]		+	*ERBB2*
9	m	2.22 [a]	20	5x0.5		0.99 [c]		+	*Unkown*
10	sm	2.21 [a]	20	9x0.5		1.27 [c]			
11	sm	1.57 [b]	15	5x0.5		1.09 [c]	*FGFR2, MYC*		
12	mp	11.7 [a]	<10	8x1		0.99 [c]			
13	ss	7.00 [a]	<10	4x3	*EGFR*	0.92 [c]			
14	sm	1.60 [b]	<10	3x0.5		0.98 [c]			
15	sm	1.60 [b]	<10	2x0.5		0.77 [c]			
16	sm	1.38 [b]	<10	3x2	*EGFR, CCND1*	1.11 [c]		+	*ERBB2*
17	mp	1.35 [b]	<10	2.0 x1.0		1.01 [c]		+	*Unknown*
18	m	0.95 [c]	<10	0.2 x 0.2		0.98 [c]			
19	ss	0.91 [c]	<10	0.5x 0.5		0.99 [c]			
20	sm	0.89 [c]	<10	0.5 x 0.5		1.01 [c]			
21	ss	21.9 [a]	> 95	10x3	*MYC*			+	*ERBB2*
22	ss	12.9 [a]	> 95	10x10					
23	ss	9.86 [a]	> 95	20x5				+	*ERBB2*
24	ss	9.20 [a]	> 95	25x10	*CCND1*				
25	ss	8.18 [a]	> 95	15x10				+	*ERBB2*
26	ss	7.87 [a]	> 95	13x10	*MYC*			+	*ERBB2*
27	ss	5.89 [a]	> 95	11x6	*EGFR*				
28	sm	5.04 [a]	> 95	24x0.5					
29	ss	4.45 [a]	> 95	9x5				+	*Unkown*
30	m	3.99 [a]	> 95	10x0.5					
31	m	3.79 [a]	> 95	10x1					
32	ss	2.28 [a]	> 95	15x15				+	*ERBB2*
33	m	1.98 [b]	> 95	4x1					
34	mp	1.87 [b]	> 95	18x2				+	*ERBB2*
35	m	1.77 [b]	> 95	2x0.5					
36	m	1.62 [b]	> 95	27x1					
37	ss	2.58 [a]	> 95	2x1				+	*ERBB2*
38	m	1.41 [b]	> 95	4x0.5					
39	m	1.39 [b]	> 95	4x0.5					
40	mp	10.7 [a]	80	8x6				+	*ERBB2*
41	m	8.62 [a]	80	5x1					
42	m	4.75 [a]	80	10x1					
43	si	2.97 [a]	80	8x5				+	*ERBB2*
44	ss	2.55 [a]	80	mosaic	*MYC*				
45	m	1.44 [b]	80	15x0.5				+	*ERBB2*
46	sm	3.78 [a]	60	15x1					
47	m	6.36 [a]	35	4x1	*EGFR, MYC, CCND1*				
48	mp	1.51 [b]	20	mosaic	*MYC*			+	*Unkown*
49	m	1.03 [c]	20	0.5x0.5					
50	m	1.07 [c]	15	0.2x0.2					
51	ss	1.54 [b]	<10	1.0x0.5	*EGFR*				

Abbreviations: ss, subserosa; sm, submucosa; se, serosa exposed; mp, mucosa propriae; m, mucosa; si, serosa infiltrating; M, metastasis; +, positive

MLPA values >2.0 were defined as amplified [a], between 1.3 and 2.0 were defined as gain [b], and between 0.7 and 1.3 were defined as normal [c].

Table 2. Gene amplifications detected by MLPA and FISH

Genes		*EGFR*	*MET*	*MYC*	*FGFR2*	*CCND1*	*CDK4*	*MDM2*	*ERBB2*	*TOP2A*
Chromosomal locus		7p11.2	7q31.2	8q24.13	10q26	11q13.3	12q14.1	12q15	17q12	17q21.2
ERBB2 amplification	Amplification	2 (2)	0	4 (4)	2 (2)	3 (3)	1 (0)	0	32(32)	8
Positive tumors	Gain	5 (3)	2 (1)	8 (3)	0	1 (0)	1 (0)	2 (0)	14 (14)	15
n=51	Normal	44 (2)	49	39	49	47	49	49	5 (5)	28
ERBB2 amplification	Amplification	0	0	0	1 (1)	0	0	1 (1)	0	0
Negative tumors	Gain	0	1 (0)	2 (1)	2 (2)	0	1 (1)	1 (0)	0	0
n=40	Normal	40	39	38	37	40	39	38	40	40

Figures in parentheses are the numbers of tumors with gene amplification detected by FISH.

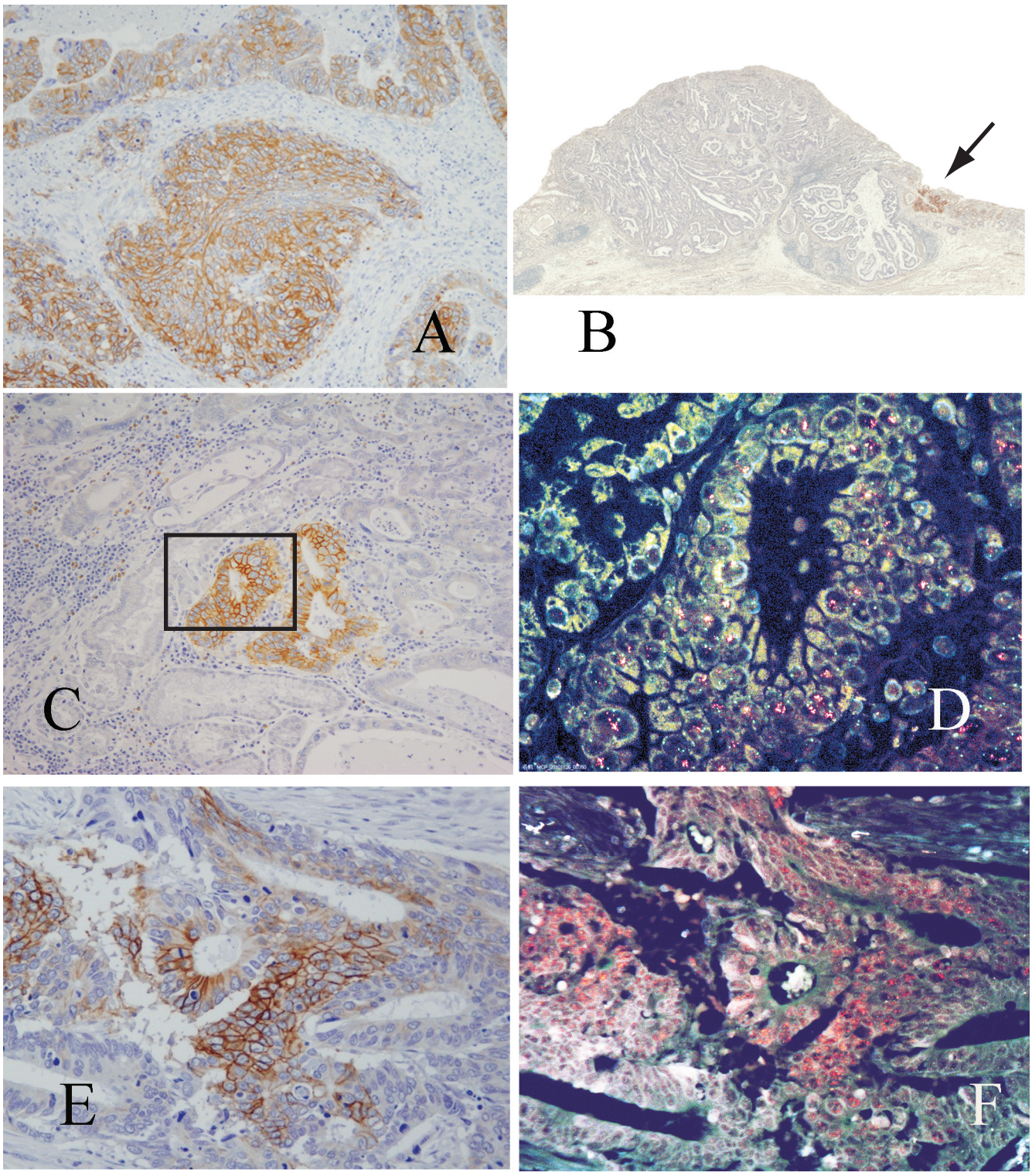


Fig. 1

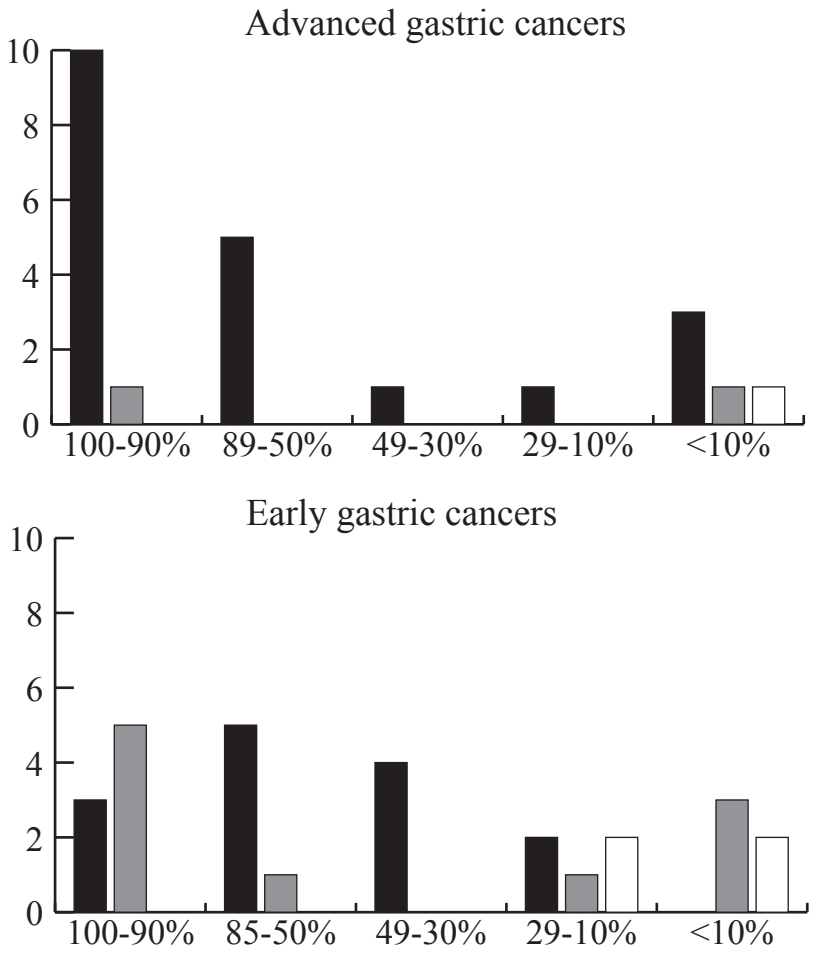

Advanced gastric cancers
10
8
6
4
2
0
100-90%
89-50%
49-30%
29-10%
<10%
Early gastric cancers
10
8
6
4
2
0
100-90%
85-50%
49-30%
29-10%
<10%

Fig. 2

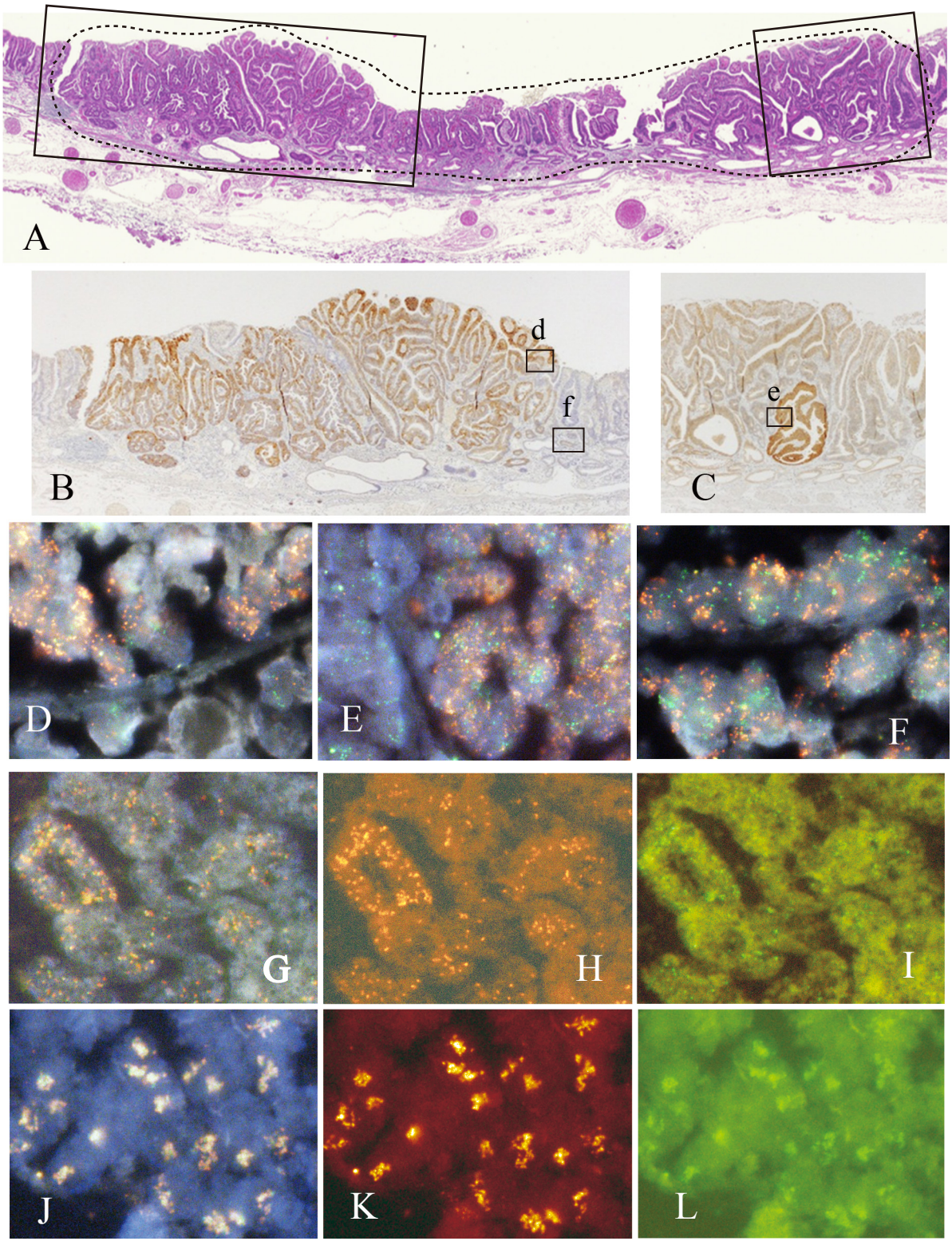


Fig. 3

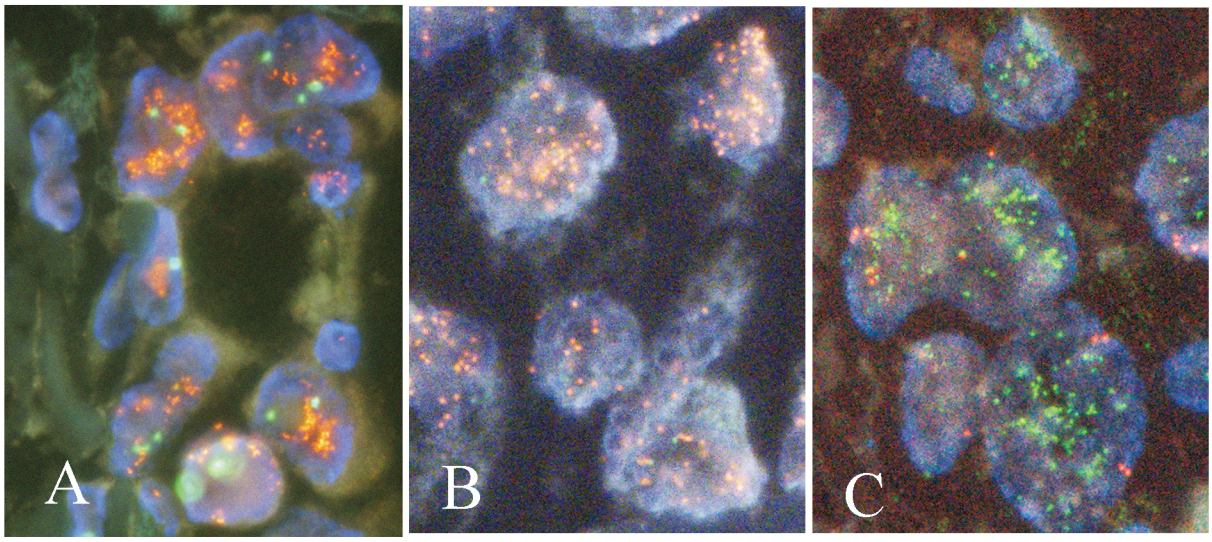
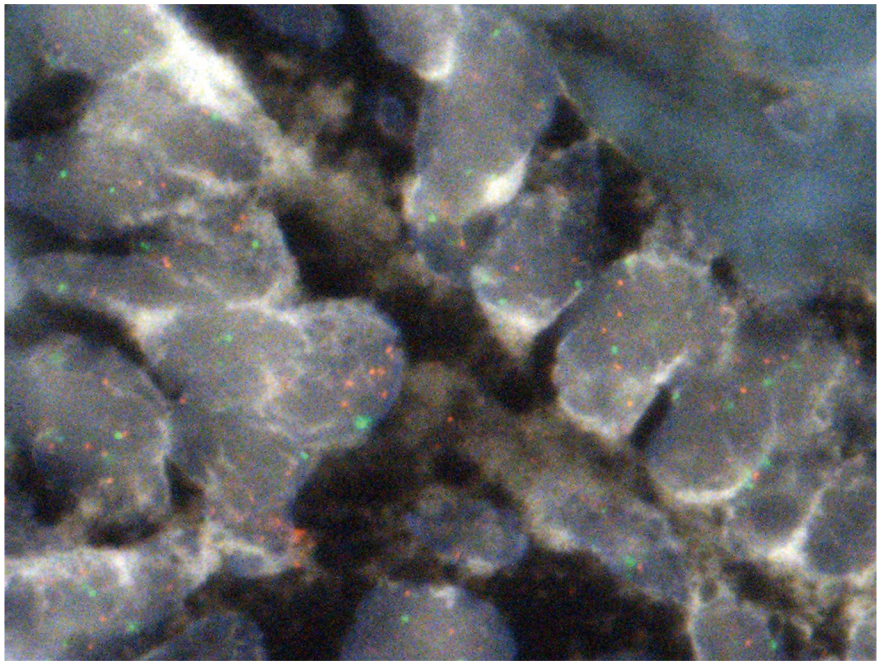
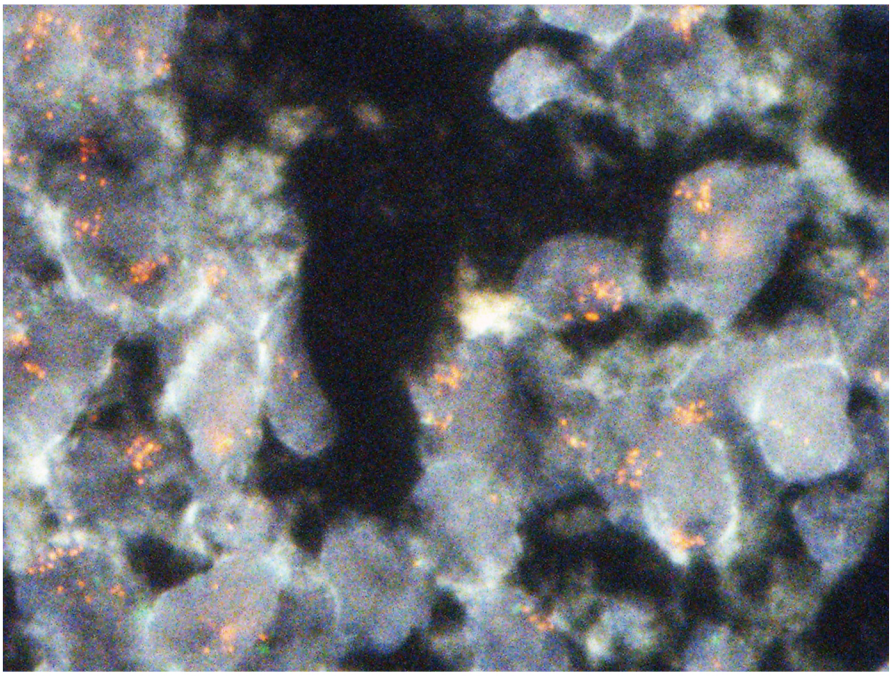


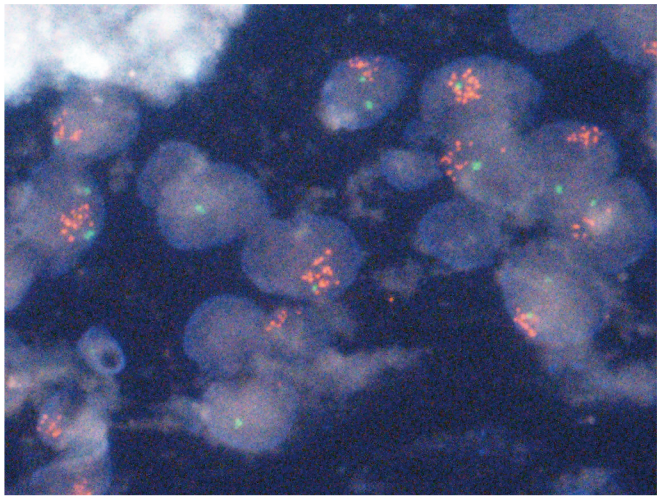
Fig. 4



Sup. Fig. 1



Sup. Fig. 2



Sup. Fig. 3