

Semi-comprehensive analysis of gene amplification in gastric cancers using multiplex ligation-dependent probe amplification and fluorescence *in situ* hybridization

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The prognosis of patients with gastric carcinomas at an advanced stage still remains dismal, and therefore novel therapeutic modalities are urgently needed. Since the successful targeting of amplified *ERBB2* with a humanized monoclonal antibody, the amplified genes of other receptor tyrosine kinases such as *EGFR*, *FGFR2*, and *MET*, as well as those of other cell regulator genes, are being considered as candidate targets of molecular therapy. The aim of the present study was to determine the amplification status of 26 genes, which are frequently amplified in solid cancers, in advanced gastric cancers. A total of 93 formalin-fixed and paraffin-embedded advanced gastric cancer tissues were examined by multiple ligation-dependent probe amplification, and 32 cases with 'gain' or 'amplified' status of 16 genes were further examined for the respective gene amplification by fluorescence *in situ* hybridization (FISH) and for the respective protein overexpression by immunohistochemistry. The frequencies of gene amplifications in advanced gastric cancers were as follows: *ERBB2* (13 cases, 14%), *FGFR2* (7 cases, 8%), *MYC* (7 cases, 8%), *TOP2A* (7 cases, 8%), *MET* (4 cases, 4%), *MDM2* (4 cases, 4%), *CCND1* (3 cases, 3%), *FGF10* (2 cases, 3%), and *EGFR* (1 case, 1%). Amplification of the receptor tyrosine kinases genes occurred in a mutually exclusive manner except for one tumor in which *ERBB2* and *FGFR2* were both amplified but in different cancer cells. Co-amplification of *ERBB2* and *MYC*, and *EGFR* and *CCND1*, in single nuclei but on different amplicons, was confirmed in one case each. Attempts at correlating the FISH status with the immunohistochemical staining pattern showed variable results from complete concordance to no correlation. In conclusion, combination of multiple ligation-dependent probe amplification and FISH analysis is a feasible approach for obtaining the semi-comprehensive genetic information that is necessary for personalized molecular targeted therapy.

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Gastric cancer is the fourth most common malignancy and ranks second among all cancer deaths worldwide.¹ According to the Japanese Research Society for Gastric Cancer, gastric cancers are divided into two groups: early gastric cancers that are confined to the mucosa and submucosa, and

advanced gastric cancers that penetrate the muscle layer or beyond.² Estimated proportions of surviving Japanese patients with 'early gastric cancer' and 'advanced gastric cancer' in the 5-years after surgery are 98.5 and 51.7%, respectively.³ Thus, novel therapeutic modalities are urgently needed for the treatment of advanced gastric carcinomas.

In 2009, an open-label, international phase 3, randomized controlled trial (ToGA, Trastuzumab for gastric cancer) found that the addition of trastuzumab, a humanized monoclonal antibody that binds to the extracellular juxtamembrane domain of a receptor tyrosine kinase, *ERBB2*, (otherwise known

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as the human epidermal growth factor receptor 2), to cisplatin-based chemotherapy significantly improved the overall survival of advanced gastric cancer patients with overexpressed and/or amplified *ERBB2* as compared with chemotherapy alone.⁴ To date, targeting *ERBB2*-overexpression/amplification remains the only successful and approved targeted therapy for gastric cancer. However, there is an urgent need for other inhibitors to be investigated for the therapy of many of the unique molecular subtypes of gastric cancer.⁵ In particular, *FGFR2* and *MET*, which encode RTKs similar to *ERBB2*, are considered as strong candidate targets because their amplification is associated with advanced stages,^{6,7} and furthermore, the amplification of *FGFR2* was preferentially found in a poorly differentiated subtype.^{8,9} In preclinical studies, cells with amplified *FGFR2* or *MET* showed overexpression of their respective proteins, and inhibitors to these receptors were shown to effectively block their downstream signal transduction and induce apoptosis.^{10–14} At present, apart from *ERBB2* targeting, approaches for targeting *MET* and *FGFR2* are the most clinically advanced of prospective targeted therapy. However, the prevalence of amplification of each of these genes, as assessed by oligonucleotide array comparative genetic hybridization, real-time PCR, fluorescence *in situ* hybridization (FISH), or silver *in situ* hybridization is low, ie, from 2 to 5% for *FGFR2*,^{9,10,12,14} and from 2 to 8% for *MET*, respectively.^{6,7,14,15} Intriguingly, a recent comprehensive genome-wide analysis of copy number alterations using a single nucleotide polymorphism array¹³ showed that amplification of *FGFR2*, *EGFR*, *ERBB2*, and *MET* occurred mutually exclusively and that other genes such as *MYC* and *CCND1* were also amplified in gastric cancer. When considering promising targets of molecular therapy, the apparently low prevalence of these gene amplifications in gastric cancer makes selection of the right set of patients that can benefit from targeted therapy a difficult challenge.

Multiplex ligation-dependent probe amplification is a new, high-resolution method for detecting numerous copy number variations in genomic sequences in a single reaction and requires only small amounts of DNA extracted from formalin-fixed paraffin-embedded tissues.^{16,17} This technique makes possible the simultaneous detection of the amplification status of *ERBB2*, *EGFR*, *MET*, *MYC*, *CCD1*, and *MDM2*.¹⁶

The aims of the present study were to determine the gene amplification status of RTKs and other cell regulator genes including *ERBB2*, *EGFR*, *FGFR2*, *MET*, *MYC*, *CCND1*, and *MDM2* in advanced gastric cancers with a view toward future introduction of molecular targeted therapy, and to examine the usefulness of multiplex ligation-dependent probe amplification in the semi-comprehensive detection of these gene amplifications.

Materials and methods

Patients

A total of 93 advanced gastric adenocarcinoma patients out of 262 gastric cancer patients who underwent surgery at the Department of Surgery in Kanazawa University Hospital between 2011 and 2013 were examined. Cancer staging was performed according to the TNM cancer staging system of the American Joint Committee of Cancer.¹⁸ The World Health Organization Classification of Tumors¹⁹ was used to determine histological classification 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 Medical Ethics Committee of Kanazawa University (Approval No 181), and written informed consent was obtained from all patients. Serial sections cut from representative formalin-fixed and paraffin-embedded cancer tissues were used for hematoxylin-eosin staining, multiplex ligation-dependent probe amplification, FISH, and immunohistochemistry.

Multiplex Ligation-Dependent Probe Amplification

A cancer area was selected on a 6 μm -thick representative tumor section, with reference to the adjacent hematoxylin-eosin staining section, taking care that non-neoplastic cells were excluded as much as possible. DNA was extracted manually from each section using proteinase K (Rosch Diagnostics, Mannheim, Germany) according to the manufacturer's (MRC-Holland, Amsterdam, The Netherlands) protocol. By using this protocol, >1 μg of sample DNA with an OD₂₆₀:OD₂₈₀ ratio within 1.1–1.7 was obtained from each tumor. DNA from the cell lines MKN7, A431, KATOIII, HSC39, and MKN45, which were previously shown to display amplified *ERBB2*, *EGFR*, *FGFR2*, *FGFR2* and *MYC*, and *MET*, respectively, were used as positive controls.^{8,20}

Multiplex ligation-dependent probe amplification analysis was performed by using two kits from MRC-Holland. The SALSA MLPA KIT P175-A2 Tumor-Gain kit contains two or three probes for each of 24 genes including *ERBB2*, *EGFR*, *MET*, *MYC*, *CCND1*, *MDM2*, and *TOP2A*. The SALSA MLPA probemix P231-A2 FGF10-FGFR2, which was originally used for the diagnosis of autosomal dominant lacrimoauriculodentodigital syndrome,²¹ contains five probes each for *FGFR2* and *FGF10*. The PCR products were separated on an ABI-310 capillary sequencer (Applied Biosystems, Foster City, CA, USA) 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.

Table 1 Genes with increased copy number ('amplified' or 'gain') detected by multiplex ligation-dependent probe amplification in advanced gastric cancer

Name of gene	MDM4	FCF10	EGFR	MET	SMO	BRAF	FGFR1	MYC	FGFR2	CCND1	CDK4	MDM2	ERBB2	TOP2A	AURKA	AR
Chromosomal locus of gene	1q32.1	5p13	7p11.2	7q31.2	7q32.3	7q34	8p11.23	8q24.13	10q26	11q13.3	12q14.1	12q15	17q12	17q21.2	20q13.31	Xq12
FISH probe	RP11-430C7	RP11-273G19	RP11-339F13	RP11-75I20	RP11-152K21	RP11-1065D4	RP11-148D21	RP11-440N18	RP11-62L18	RP11-300I6	RP11-571M6	RP11-775J10	RP11-62N23	RP11-480O10	RP11-75K20	RP11-80F19
No. of 'amplified' tumors	0	2 (2)	1 (1)	1 (1)	0	0	0	3 (3)	4 (4)	3 (3)	0	1 (1)	11 (11)	4 (4)	0	0
No. of 'gain' tumors	8 (0)	5 (1)	5 (0)	4 (3)	2 (0)	16 (0)	2 (0)	10 (6)	10 (5)	0 (0)	2 (0)	19 (3)	5 (3)	13 (4)	15 (0)	21 (0)

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

Average 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.^{16,17}

FISH

Tumors with 'amplified' or 'gain' of genes as determined by multiplex ligation-dependent probe amplification were further examined using FISH to validate the accuracy of the multiplex ligation-dependent probe amplification results for the respective gene amplification. FISH probes acquired from BACPAC Resources (Oakland, CA, USA) are summarized in Table 1. The probes were labeled with SpectrumOrange or SpectrumGreen using a nick translation kit (Abbott Laboratories, Abbott Park, IL, USA). 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. Tumors exhibiting co-amplification of different genes were further examined by simultaneous hybridization with two probes specific to the genes that were labeled with different fluorescent markers to determine the coexistence of the amplified genes in single cells and single amplicons.

Removal of protein from the tissue sections, denaturation, hybridization, and post-hybridization washing were performed as described previously.¹⁷ The tissue sections were counterstained with DAPI II (Abbott) and examined using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Triple Bandpass Filter set (Abbott) for DAPI II, SpectrumOrange and SpectrumGreen, and a filter set specific for SpectrumOrange or SpectrumGreen. FISH images were recorded through a cooled charge-coupled device camera (DP-70, Olympus) linked to a computer software program (DP Manager, Olympus).

Scoring and evaluation of FISH slide were performed manually by counting gene signals and the centromere signals, on which the gene was located, of 20 tumor nuclei per case. The average copy number of the gene and the centromere, and their ratio, were determined for each case. Gene amplification was determined according to the American Society of Clinical Oncology/College of American Pathologists-approved criteria of *ERBB2* amplification, except that the quantity of positive cells was defined as more than six gene copies per nucleus or gene signals/centromere signals >2.2.²² In addition, low-level amplification was arbitrarily defined as gene signals/centromere signals >2.2 and gene copy number of <6, and high polysomy was arbitrarily defined as gene signals/centromere signals <1.8 and gene copy number >6. In cases of apparent intratumoral heterogeneity in gene amplification, the fractions (%) of the tumor cells containing gene

Table 2 Antibodies and dilutions applied for immunohistochemistry

Antibody	Clone	Dilution	Antigen retrieval	Manufacturer
ERBB2	Polyclonal	1:400	None	Nichirei, Tokyo, Japan
EGFR	EGFR.25	1:100	PC pH6, citrate	Novocastra Lab, Newcastle, UK
FGFR2	ab58201	1:1000	None	Abcam, Cambridge, UK
MYC	Y69	1:50	PC pH9, EDTA	Pharmingen, San Diego, CA, USA
MDM2	IF2	1:40	PC pH6, citrate	Calbiochem, La Jolla, CA, USA
MET	SP44	1:50	PC pH6, citrate	Spring Bioscience, Pleasanton, CA, USA
CCND1	SP4	Prediluted	PC pH6, citrate	Nichirei, Tokyo, Japan

Abbreviation: PC, pressure cooking.

amplification were scored using broad tiers of < 30, 30–70, and > 70%.

Immunohistochemistry

Immunohistochemistry for ERBB2, EGFR, FGFR2, MET, MYC, CCND1, and MDM2 was performed on tumors with FISH-proved amplification. The antibody clones, manufacturers and dilutions, and the antigen retrieval methods are summarized in Table 2. Antibody binding was visualized using the LSAB system (Dako, Glostrup, Denmark). For evaluation of positive staining of ERBB2, EGFR, FGFR2, and MET, each tumor, or portion of tumor, was scored 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. For evaluation of MYC, CCND1, and MDM2 staining, only nuclear immunostaining, which was significantly higher than that of control cells of normal gastric mucosa, was considered as positive.

Statistical Analyses

The association of gene amplification and histology of the tumor, and association between amplifications of different genes were analyzed for significance using either Fischer's exact probability test or the χ^2 -test.

Results

Multiplex Ligation-Dependent Probe Amplification

Multiplex ligation-dependent probe amplification analyses were successfully performed on all 93 of the formalin-fixed and paraffin-embedded advanced gastric adenocarcinoma tissue samples. The gene status of the 26 genes analyzed in the 93 tumors were categorized as 'amplified', 'gain', or 'normal' based on the mean multiplex ligation-dependent probe amplification peak values. There were no cases with 'lost' genes. The number of tumors with 'amplified' or 'gain' of specific genes by multiplex ligation-dependent probe amplification analysis is summarized in

Table 1. All tumors displayed a normal copy number of *MYCN*, *ALK*, *PDGFRA*, *KIT*, *KDR*, *DHFR*, *ABL1*, *RET*, *CCND2*, and *AURKB*.

FISH

Out of the total 93 advanced gastric cancers, 32 tumors (34%) displayed gene amplification of one of *FGFR10*, *EGFR*, *MET*, *MYC*, *FGFR2*, *CCND1*, *MDM2*, *ERBB2*, or *TOP2A*. In FISH analysis, most amplifications appeared as either tightly clustered or numerous scattered signals of the amplified genes, suggesting amplicons in homogeneously staining regions and double minute chromosomes, respectively.²³ Cancer nuclei with colocalization of an amplified gene and the amplified centromeric lesion on which the gene is located, which results in a yellow fusion signal²⁴ as shown in Supplementary Figure 1, was rarely observed. Approximately half of the gene amplifications occurred in an intratumoral heterogeneous fashion.

All tumors with genes that were categorized as 'amplified' by multiplex ligation-dependent probe amplification had cancer cells with gene amplification that could be detected by FISH as shown in Table 1. The tumors with 'gain' of *MDM2*, *SMO*, *BRAF*, *FGFR1*, *CDK4*, *AURKA*, and *AR* by multiplex ligation-dependent probe amplification showed polysomy of the chromosome on which the target gene is located; however, amplification of these genes was not found.

Table 3 describes the clinicopathological characteristics of the 32 tumors with gene amplification and indicates the fraction (%) of the amplified cells and the type of amplification in each case. Three quarters (24 of 32) of the amplifications were of genes encoding RTKs: representative FISH images of amplified *ERBB2*, *FGFR2*, *MET*, and *EGFR* were shown in Figure 1a and b, Figures 1a and 2a, Figure 3a and c, and Figure 4a, respectively. Except for Case 7, amplification of these RTK genes occurred in a mutually exclusively manner, ie, each tumor had a single RTK gene mutation. In Case 7, dual-color FISH analysis demonstrated 'homogeneously staining region'-type *ERBB2* amplification and 'double minute chromosome'-type *FGFR2* amplification intermingled in a mosaic pattern. However, no single cell

Table 3 Advanced gastric cancers with gene amplification

Case No.	Histo-logical classification	Anatomic stage /prognostic groups	Neoajduvant therapy	FISH findings of amplified genes									
				ERBB2	TOP2A	FGFR2	MET	EGFR	MYC	MDM2	CCND1	FGF10	
1	Tub	IV		HSR (>70)									
2	Tub	IV	DCS	HSR (>70)	HSR (>70)								
3	Por	IV	DCS/ Herceptin	DM (>70)									
4	Tub	IV		Low amp (30-70)							HSR (30-70)		
5	Tub	IV		HSR (>70)						Low amp (>70)			
6	Tub	IV	DCS	HSR (>70)	Low amp (>70)								
7	Tub	IIIc	DCS	HSR (30-70)		DM (30-70)							
8	Tub	IIIB	DCS	DM (>70)	DM (30-70)								
9	Tub	IIIB	S-1	Co-amp HSR (>70)	co-amp HSR (>70)					High poly (>70)			Low amp (>70)
10	Tub	IIIA		Low amp (>70)	Low amp (>70)								
11	Tub	IIIA		HSR (>70)	HSR (>70)								
12	Tub	IIA		Co-amp HSR (>70)	co-amp HSR (>70)					HSR (>70)			
13	Tub	IIA		HSR (30-70)									
14	Por	IV				DM (30-70)							
15	Por	IV				High poly (30-70)						HSR (30-70)	
16	Por	IIIB	DCS			DM (30-70)							
17	Por	IIIA				DM (<30)				DM (<30)			
18	Por	IIA				HSR (>70)							
19	Tub	IIA				DM (30-70)							
20	Por	IV	DCS				Low amp (>70)						
21	Tub	IV	DCS				DM (30-70)						
22	Por	IV	DCS				HSR (30-70)						
23	Muc	IIIA					HSR (30-70)						
24	Tub	IIIA						DM (>70)				HSR (>70)	
25	Por	IV	DCS							HSR (>70)			
26	Por	IV								DM (>>HSR (30-70))			HSR (>70)
27	Por	IIIB	S-1/DOC							DM (30-70)			
28	Pap	IIIB								HSR (30-70)			
29	Por	IIIA	DCS									Low amp> DM (>70)	
30	Tub	IV								Low amp (>70)			
31	Por	IIA	DCS							DM (30-70)			
32	Mixed	IIIA	DCS										Low amp (>70)
Numbers of cases with gene amplification				13	7	7	4	1	7	4	3		2
Fraction (%) of cases with gene amplification				14	8	8	4	1	8	4	3		3

Abbreviations: Co-amp HSR, co-amplification of a gene and the centromeric lesion in HSR; DCS, combination chemotherapy with docetaxel cisplatin, and S-1; DM, double minute chromosome type amplification; DOC, docetaxel; high poly, high polysomy; HSR, homogeneously staining region type amplification; low amp, low amplification.; mixed, mixed carcinoma; muc, mucinous carcinoma; pap, papillary adenocarcinoma; Por, poorly cohesive adenocarcinoma; Tub, tubular adenocarcinoma. Numbers in parentheses indicate % of the amplified cells in each case.

had amplification of both genes, as shown by dual-color FISH in Figure 1a. *MYC* was amplified in seven tumors, and was co-amplified with *ERBB2* in three tumors. On the basis of dual-color FISH analysis, co-amplification of *ERBB2* and *MYC* in single nuclei but on different amplicons was confirmed in case 9 (Figure 1b). *MDM2* amplification was found in four tumors; one showed co-amplification with *ERBB2* and the other showed co-amplification with the *FGFR2*; however, cancer cells with different gene amplification occupied different tumor areas as shown in Figure 2. *CCND1* was amplified in three tumors; one showed co-amplification with *EGFR* and the other with *FGFR2*. Dual-color FISH analysis could confirm co-amplification of *EGFR* and *CCND1* in single nuclei but on different amplicons in case 24 (Figure 4a). All of the tumors with *TOP2A* amplification were also positive for *ERBB2* amplification. Furthermore, dual-color FISH showed that *TOP2A* signals and *ERBB2* signals were closely associated (Supplementary Figure 2).

Immunohistochemistry. Immunohistochemistry for *ERBB2* indicated 11 tumors with 3+ or 2+ staining (Figure 1e), one tumor with 1+ staining (Case 7) (Figure 1c) and one tumor (case 10) was negative. Immunohistochemistry for *FGFR2* displayed a variety of cytoplasmic staining from weak to strong, which was found to correspond to *FGFR2*-amplified tumor cells (Figures 1d and 2d). However, similar cytoplasmic staining was also commonly found in non-*FGFR2*-amplified cancer cells (Figure 2a, b, and d) and occasionally in non-neoplastic glandular cells. Immunohistochemistry of *MET* showed inconsistent results; 3+ staining in Case 21 (Figure 3b), 2+ staining in Cases 22 and 23, and negative staining in Case 20. In Case 21, the *MET* amplification-positive and -negative cells were randomly intermingled; even single cancer tubules were composed of amplification-positive and -negative cells. FISH analysis of serial sections showed that the *MET*-amplified cells corresponded to *MET* 3+ immunostained cells, and that non-*MET*-amplified cells corresponded to *MET* immunohistochemically-negative cells as shown in Figure 3a and b. In Case 22, *MET*-amplified and non-amplified cells were in different areas and both showed 2+ *MET* immunostaining. Thus these cells were not differentiated by immunohistochemistry as shown in Figure 3e and f. Case 24 that showed homogeneous *EGFR* amplification showed 3+ immunostaining of the *EGFR* (Figure 4b).

Most immunohistochemical results of *MYC* were equivocal (Figure 1f). In the immunohistochemistry analysis of *MDM2* and *CCND1*, scattered positive nuclear staining was found and clear coincidence of this staining with the respective amplification-positive nuclei was not confirmed (Figure 2c and e, Figure 4a and c, respectively).

Clinicopathological Findings. Amplification of *ERBB2* was significantly more frequent in differentiated carcinomas than in undifferentiated carcinomas ($P=0.04$); however, the amplification of other genes showed no correlation with histological differentiation.

Discussion

In the present study, 32 of a total of 93 advanced gastric cancers (34%) displayed definite gene amplification and most of this amplification occurred in a FISH pattern that was reminiscent of cytogenetic homogeneously staining region or double minute chromosomes. Furthermore, 26% (24 of 93) of the amplifications were amplification of one of four genes (*ERBB2*, *FGFR2*, *MET*, and *EGFR*) that encode a RTK. The frequencies of these gene amplifications were not significantly different from those previously reported for *ERBB2*, *FGFR2*,^{9,10,12,14} *MET*,^{6,7,14,15} and *EGFR*.^{6,20} It is intriguing that, except for case 7, RTK gene amplification occurred in a mutually exclusive manner. Even in Case 7, RTK gene co-amplification did not occur in the same cells. Thus, if a common tyrosine kinase inhibitor to the four RTKs were available, 24 of 93 (26%) cases of advanced gastric cancer would potentially be susceptible to that agent. This characteristic of RTKs is one of the reasons why RTK gene amplifications have attracted much attention as molecular targets even though the prevalence of individual RTK gene amplification is low.^{6,12,13}

However, if small fractions of amplified cells that cannot be detected by screening with multiplex ligation-dependent probe amplification and/or early gastric cancers are included, co-amplification of RTK genes is not exceptional in gastric cancers. On the contrary, our previous study showed a marginal trend for co-amplification of *EGFR* and *ERBB2*; however, this trend was not statistically significant.²⁵ In our more recent study in which we examined the co-amplification of *ERBB2* and other RTK genes, we found that of 51 tumors with *ERBB2* amplification, 14% (7/51) of the tumors displayed co-amplification with *EGFR*, one tumor showed co-amplification with *FGFR2*, and one tumor showed co-amplification with *MET* and *FGFR2*.¹⁶ It is probable that redundancy of RTKs, which are unnecessary for cancer cell growth, diminishes in the course of gastric cancer progression.

In contrast to the lack of co-amplification of RTK genes, *MYC*, and RTK genes are occasionally co-amplified in single cancer cells. In the present study co-amplification of *MYC* and *ERBB2* in the same cell was found in three tumors, and, in our previous study, co-amplification of *MYC* and *ERBB2* or *EGFR* occurs in a non-incident manner.²⁵ Thus, these co-amplifications may be of some merit for survival of cancer cells. A similar selective co-amplification of *ERBB2* and *MYC* was found in breast cancer cells

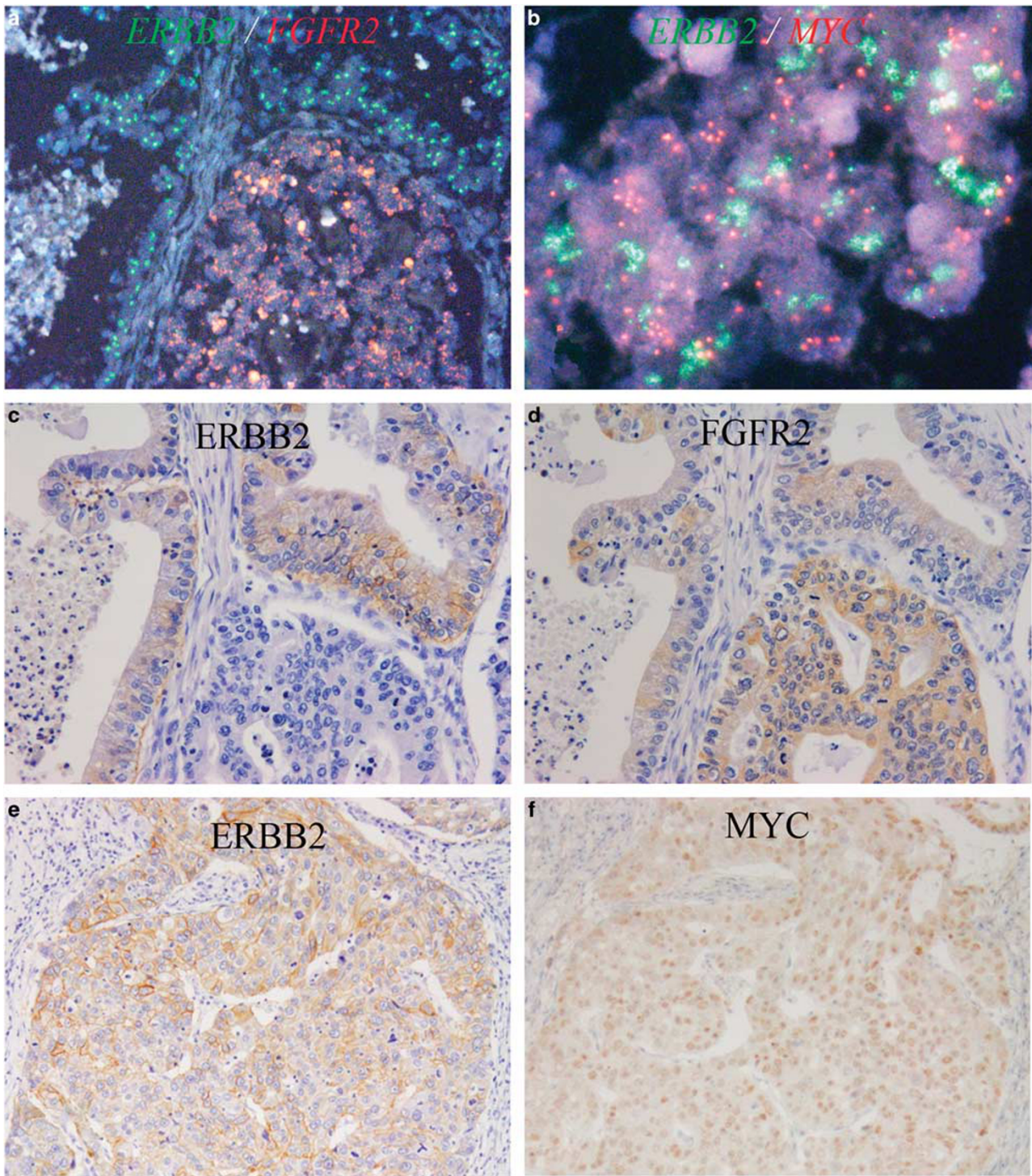


Figure 1 Gastric cancers with co-amplification of *ERBB2* and *FGFR2* (Case 7), and *ERBB2* and *MYC* (Case 9). In Case 7, amplification of *ERBB2* and *FGFR2* was found in different cancer cells. In Case 9, *ERBB2* and *MYC* was in the same nuclei. Gene amplifications were analyzed by dual-color FISH (**a**: green fluorescence, *ERBB2*; orange fluorescence, *FGFR2*) (**b**: green fluorescence, *ERBB2*; orange fluorescence, *MYC*) and their protein overexpression was analyzed by immunohistochemistry (**c** and **e**, *ERBB2*; **d**, *FGFR2*; **f**, *MYC*). Panels **a**, **c**, and **d** show the same field. *ERBB2*- and *FGFR2*-amplified cells in panel **a** correspond to the 1+ *ERBB2*-stained cells in panel **c** and the cells with weak cytoplasmic staining of *FGFR2* in panel **d**, respectively. The cells with gene amplification in panel **b** showed 2+ immunostaining of *ERBB2* and equivocal nuclear staining of *MYC*.

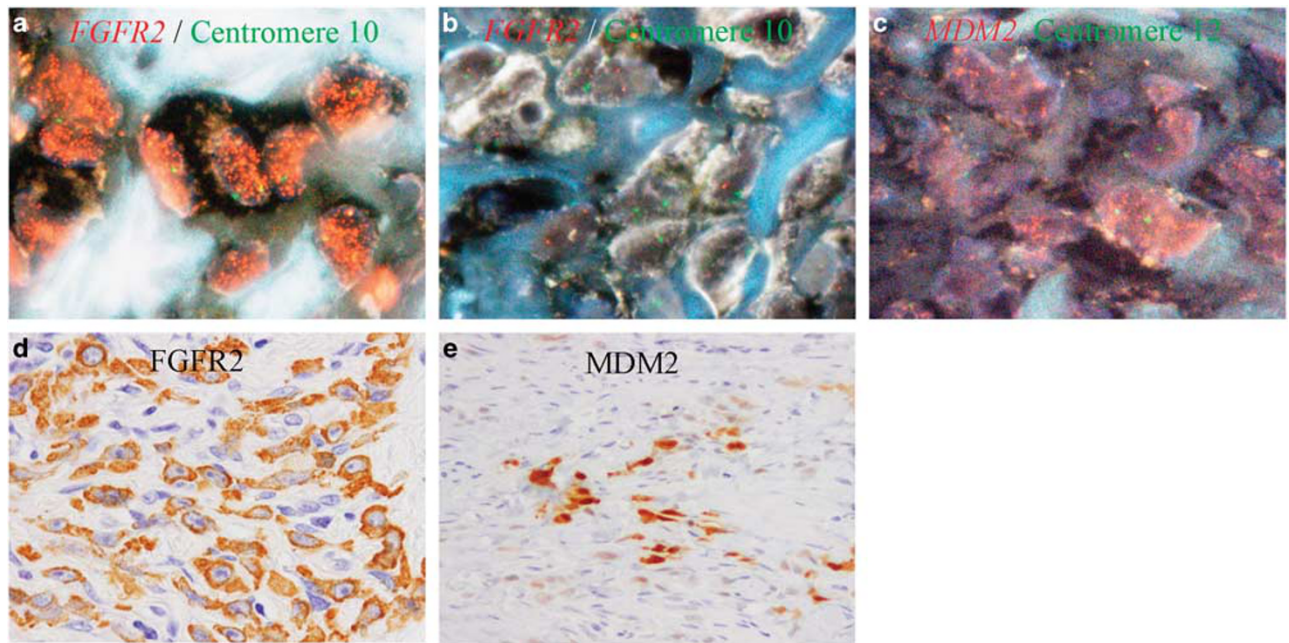


Figure 2 A poorly cohesive-type gastric cancer with *FGFR2*-amplified and *MDM2*-amplified cancer cells in different areas (Case 17). *FGFR2* and *MDM2* gene amplification was analyzed by dual-color FISH (a and b: orange fluorescence, *FGFR2*; green fluorescence, centromere 10. c: orange fluorescence, *MDM2*; green fluorescence, centromere 12) and their protein overexpression was analyzed by immunohistochemistry (d, *FGFR2*; e, *MDM2*). Panels a and b are different fields of the same area of panel d. Intense cytoplasmic staining of *FGFR2* (d) was detected in both *FGFR2*-amplified (a) and *FGFR2*-non-amplified (b) cells. Panels c and e are the same area. Most but not all *MDM2*-amplified cells show 3+-nuclear staining of *MDM2*.

and it has been speculated that this co-amplification might be a very favorable prognostic factor when combined with adjuvant therapy by trastuzumab.^{26,27} However, this hypothesis was recently refuted. Instead, new data have shown that this co-amplification is associated with poor outcome and is associated with the sequence of acquisition of a malignant stem cell-like phenotype that maintains lower growth and therefore may be a key target of new therapy.²⁸

TOP2A resides very close to *ERBB2* on the chromosome and its amplification was only observed with the concomitant amplification of *ERBB2*. However, *TOP2A* is not simply a 'passenger' gene because *TOP2A* encodes an enzyme, topoisomerase II α , which is required for DNA replication. Moreover, clinical studies have confirmed that breast cancer patients with *TOP2A* gene amplification are more sensitive to topoisomerase II α -based therapy.²⁹ Unfortunately this therapy is not commonly used for the treatment of gastric cancers in Japan.

Information regarding *CCND1* amplification is useful because expression of *CCND1* may cause resistance to cisplatin,³⁰ which was coadministered with trastuzumab in the ToGA clinical trial. To the best of my knowledge, this is the first report of amplification of *FGF10* in gastric cancer. Although stomach development is dependent on FGF10- and *FGFR2*-mediated signaling,³¹ it remains to be clarified whether *FGF10* amplification is relevant to carcinogenesis of gastric cancer through enhanced signaling. In this study an additional candidate for

molecular targeting, amplified *MDM2*, was found in *ERBB2*-non-amplified gastric cancers. *MDM2* is an oncogene whose expression controls tumorigenesis by promoting degradation of the tumor suppressor protein p53. Amplification of *MDM2* impacts cancer treatments that use selective inhibitors of *MDM2* proteins.^{32,33} Indeed, in an *in vivo* study, treatment with nutlin, an *MDM2* antagonist, induced p53-dependent transcription and apoptosis in liposarcoma cells that were positive for *MDM2* amplification.³⁴

In this study we screened advanced gastric carcinomas for possible gene amplification by multiplex ligation-dependent probe amplification and confirmed the results obtained using FISH analysis, which has been considered as the gold standard method for analysis of gene amplification. FISH probes span ~200 kb of DNA sequence, which is similar to or several-fold larger than the target gene size. In contrast, multiplex ligation-dependent probe amplification targets intragenic portions of DNA sequence with sequence sizes of 50–100 bases. Although for a typical gene amplification the result of multiplex ligation-dependent probe amplification should theoretically coincide with that of FISH, the copy number detected by multiplex ligation-dependent probe amplification was often lower than that detected by FISH owing to intratumoral heterogeneity and/or contamination of non-neoplastic DNA. In the present study, the cutoff value of 1.3 that was recommended by the multiplex ligation-dependent probe amplification kit manufacturer was

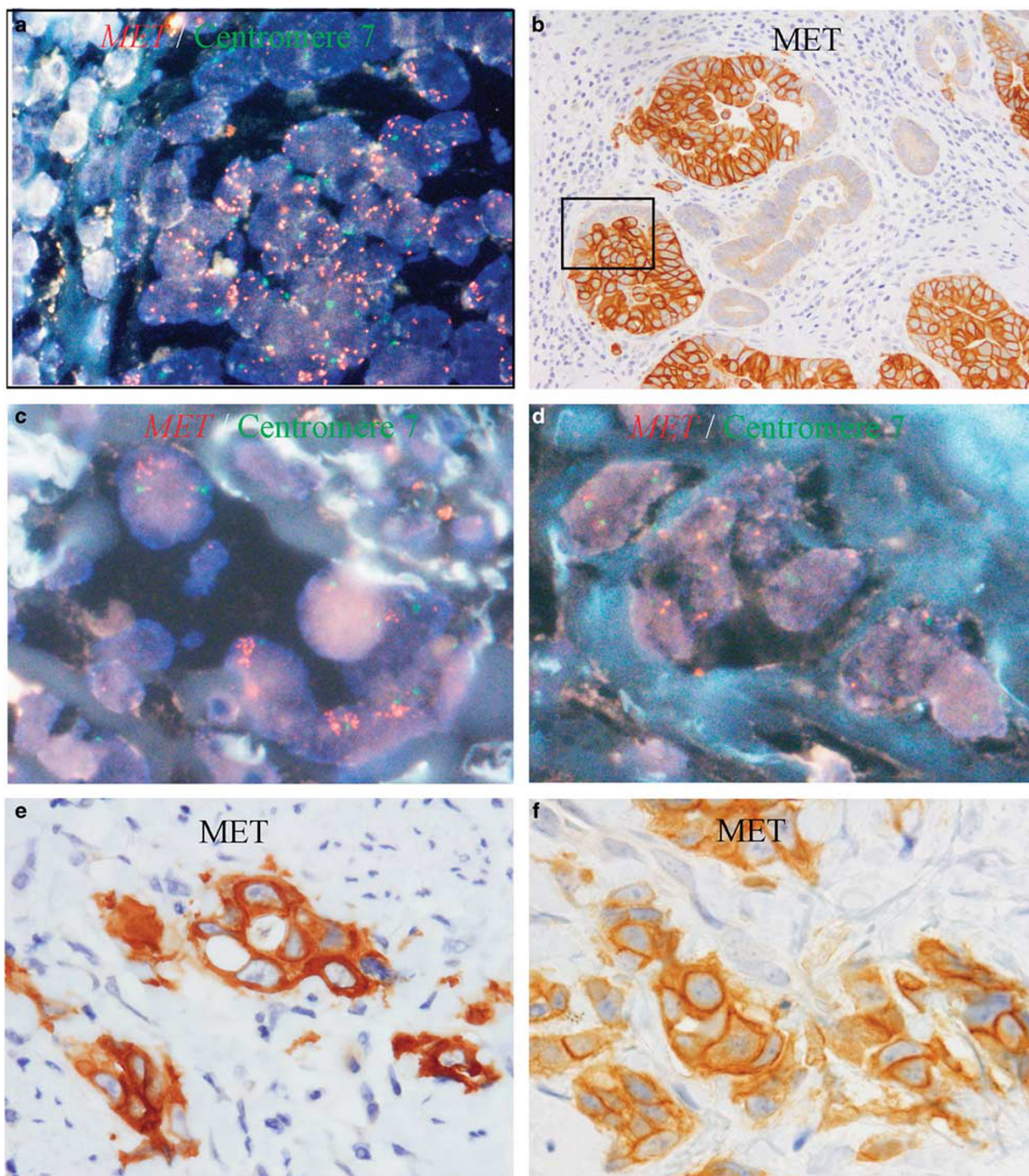


Figure 3 Amplification and overexpression of *MET* in gastric carcinomas (Cases 21 and 22). A tubular adenocarcinoma (Case 21, **a** and **b**) and a poorly cohesive-type adenocarcinoma (Case 22, **c**–**f**) were analyzed for *MET* gene amplification by dual-color FISH (**a**, **c**, and **d**: orange signals, *MET*; green signals, centromere 7) and for *MET* protein overexpression by immunohistochemistry (**b**, **e**, and **f**). In the tubular adenocarcinoma (Case 21), cancer cells with 3+ immunostaining of *MET* displayed amplified *MET*; cells with negative immunostaining displayed no amplification (**a** and **b**). The region enclosed by the black rectangle in panel **b** corresponds to the region shown in panel **a**. The poorly cohesive type adenocarcinoma (Case 22) consisted of *MET*-amplified (**c**) and non-amplified cancer cells (**d**) in different areas, and both areas displayed 2+ staining for *MET* by immunohistochemistry (**e** and **f**). Panels **c** and **e**, and panels **d** and **f** are the same field.

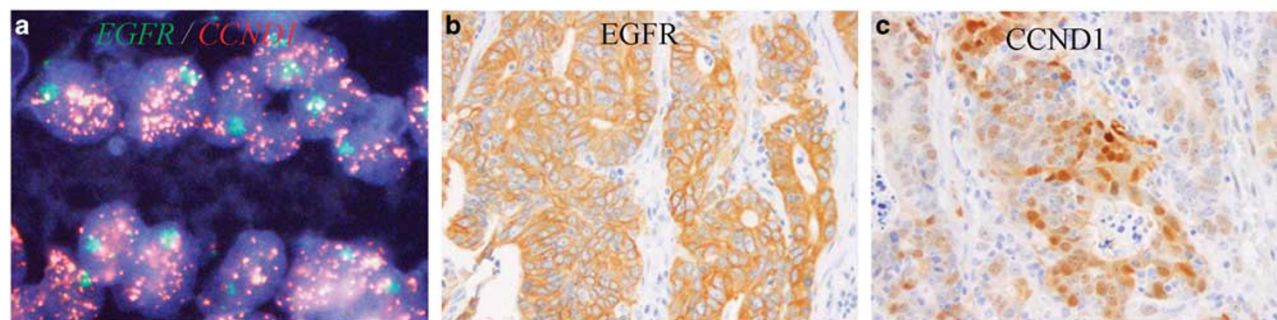


Figure 4 A gastric cancer with co-amplification of *EGFR* and *CCND1* in the same nuclei (Case 24). *EGFR* and *CCND1* gene amplification was analyzed by dual-color FISH (a: green fluorescence, *EGFR*; orange fluorescence, *CCND1*), and their protein overexpression was analyzed by immunohistochemistry (b, *EGFR*; c, *CCND1*). The gene-amplified cells showed 3+ immunostaining of the *EGFR* and scattered nuclear staining of *CCND1*.

used for all of the genes. Although all of the cases defined as ‘amplified’ by multiplex ligation-dependent probe amplification were also amplified by FISH analysis, seven genes that were defined as ‘gain’ by multiplex ligation-dependent probe amplification showed no real amplification by FISH analysis (Table 1). It may therefore be possible to increase the specificity of the test by resetting the cutoff values for these genes.³⁵ However, for the purpose of screening of gene amplification, high sensitivity precedes specificity and the cutoff value of 1.3 was very acceptable for such screening.

MYC, *CCND1*, *MDM2*, *ERBB2*, *EGFR*, *FGFR2*, and *MET* are considered to be involved in carcinogenesis through protein overexpression of amplified genes. Thus, immunohistochemistry that is familiar to most diagnostic pathologists might be the first choice for examination of these aberrations. However, our attempts to correlate FISH status with immunohistochemical staining resulted in variable correlations between the two types of measurement ranging from complete correspondence to dissociation. The protein overexpression of *MYC*, *CCND1*, and *MDM2* is regulated by several pathways and thus gene amplification may not necessarily provide a straightforward correlation with protein overexpression. As for RTK genes, the hypothesis that ‘amplification causes overexpression’ is fundamentally established for *ERBB2*, and is highly supported for *EGFR*, *FGFR2*, and *MET*.^{10–14} However, even for RTKs, inconsistent results between FISH and immunohistochemical analyses were often obtained, mostly because of inherent technical reasons of immunohistochemistry such as specificity of antibodies, fixative used, duration of fixation, staining procedures, and/or scoring system. Therefore, at the present time gene amplification provides the best biomarkers for helping to select the right patient candidate for molecular therapy targeted toward these genes, and multiplex ligation-dependent probe amplification is the most feasible method for screening these gene amplifications.

In conclusion, given the apparent low prevalence of amplification of candidate target oncogenes for therapy of gastric cancer, implementation of

semi-comprehensive screening by multiplex ligation-dependent probe amplification followed by confirmatory FISH analysis should facilitate the identification of patients who may benefit from such tailored therapy.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)