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

doi: 10.1038/modpathol.2015.33

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

Akishi Ooi [1, 2], Takeru Oyama [1], Ritsuko Nakamura [1], Ryousuke Tajiri [1], Hiroko Ikeda [2], Sachio Fushida [3], Hiroyuki Nakamura [4], Yoh Dobashi [5]

1. Department of Molecular and Cellular Pathology, Graduate School of Medical Science, Kanazawa University
2. Pathology Section, University Hospital, Kanazawa University
3. Department of Surgery, Graduate School of Medical Science, Kanazawa University
4. Department of Environmental and Preventive Medicine, Graduate School of Medical Science, Kanazawa University
5. Department of Pathology, Saitama Medical Center, Jichi Medical University

Running title: Gene amplification in gastric cancer

This work was financially supported by the Japanese Ministry of Education, Sports, Science and Culture [C2546015 (SF), C25460452 (AO), C26460438 (YD), and Young Scientists (B) (25860266) (TO) and (26860235) (RN)], and Smoking Research Foundation (YD).

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

Abstract

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 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* (one 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 were confirmed in one case each. Attempts at correlating the fluorescence *in situ* hybridization 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 fluorescence *in situ* hybridization analysis is a feasible approach for obtaining the semi-comprehensive genetic information that is necessary for personalized molecular targeted therapy.

Abbreviations: fluorescence *in situ* hybridization (FISH); receptor tyrosine kinase (RTK)

Key words: FISH, Gastric cancer, Gene amplification, Multiple ligation-dependent probe amplification, Molecular targeted therapy, RTK

Introduction

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 five-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 (RTK), ERBB2, (otherwise known as the human epidermal growth factor receptor 2, HER2), 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.[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] Currently, 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: from 2% to 5% for *FGFR2*,[9, 10, 12, 14] and from 2% to 8% for *MET*.[6, 7, 14, 15] Intriguingly, a recent comprehensive genome-wide analysis

of copy number alterations using an single nucleotide polymorphism array [13] 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 towards 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 Tumors [19] 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, Manheim Germany) according to the manufacturer's (MRC-Holland, Amsterdam, The Netherlands) protocol. By using this protocol, more than 1 μg of sample DNA with an OD_{260}:OD_{280} 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. 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) are summarized in Table 1. The probes were

labeled with SpectrumOrange™ or SpectrumGeen™ 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 co-existence 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 less than 6, and high polysomy was arbitrarily defined as: gene signals/centromere signals <1.8 and gene copy number greater than 6. In cases of apparent intratumoral heterogeneity in gene amplification, the fractions (%) of the tumor cells containing gene-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 MDM staining, only nuclear immunostaining that 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 chi-square 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 co-localization of an amplified gene and the amplified centromeric lesion on which the gene is located, which results in a yellow fusion signal [24] as shown in Suppl. Fig. 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 Figs 1A and B, 1A and 2A, 3A and 3C, and 4A respectively. Except for Case 7, amplification of these RTK genes occurred in a mutually exclusively manner i.e. 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 had amplification of both genes, as shown by dual color FISH in Fig. 1A. *MYC* was amplified in seven tumors, and was co-amplified with *ERBB2* in three tumors. Based on dual-color FISH analysis, co-amplification of *ERBB2* and *MYC* in single nuclei but on different amplicons was confirmed in case 9 (Fig. 1B). *MDM2* amplification was found in 4 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 Fig 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 (Fig. 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 (Suppl. Fig. 2).

Immunohistochemistry

Immunohistochemistry for ERBB2, indicated 11 tumors with 3+ or 2+ staining (Fig. 1E), one tumor with 1+ staining (Case 7) (Fig. 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 (Fig 1D and 2D). However similar cytoplasmic staining was also commonly found in non-*FGFR2*-amplified cancer cells (Figs. 2A, B and D) and occasionally in non-neoplastic glandular cells. Immunohistochemistry of MET showed inconsistent results; 3+ staining in Case 21 (Fig. 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 Figs. 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 Figs. 3E and F. Case 24 that showed homogeneous *EGFR* amplification showed 3+ immunostaining of the EGFR (Fig.4B).

Most immunohistochemical results of MYC were equivocal (Fig. 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 (Figs. 2C and E, Fig. 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*, [12 9, 10, 14] *MET* [6, 7, 14, 15] and the *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 detect 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-incidental 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 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 IIα, that is required for DNA replication. Moreover, clinical studies have confirmed that breast cancer patients with TO*P2A* gene amplification are more sensitive to topoisomerase IIα-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 co-administered 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 approximately 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 due to intratumoral heterogeneity and/or contamination of non-neoplastic DNA. In the present study, the cut-off value of 1.3 that was recommended by the multiplex ligation-dependent probe amplification kit manufacturer was 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 cut-off values for these genes.[35] However, for the purpose of screening of gene amplification, high sensitivity precedes specificity and the cut-off 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, due mostly to 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 towards 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.

The authors declare no conflict of interest.

Legends

Table 1.
Genes with increased copy number (‘amplified’ or ‘gain’) detected by multiplex ligation-dependent probe amplification in advanced gastric cancer. Values in parentheses are the numbers of tumors with gene amplification detected by FISH.

Table 2.
Antibodies and dilutions applied for immunohistochemistry.
Abbreviations: MW, microwave; PC, pressure cooking

Table 3.
Advanced gastric cancers with gene amplification. Numbers in parentheses indicate % of the amplified cells in each case.
Abbreviations: tub, tubular adenocarcinoma; por, poorly cohesive adenocarcinoma; pap, papillary adenocarcinoma; mixed, mixed carcinoma; muc, mucinous carcinoma; DCS, combination chemotherapy with docetaxcel, cisplatin and S-1; DOC, docetaxcel; HSR, homogeneously staining region type amplification; DM, double minute chromosome type amplification; co-amp HSR, co-amplification of a gene and the centromeric lesion in HSR; high poly, high polysomy; low amp, low amplification.

Fig. 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.

Fig. 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.

Fig. 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 immmunostaining 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.

Fig. 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.

Suppl. Fig. 1

Example of cancer nuclei with rare co-localization of an amplified gene and the amplified centromeric lesion on which the gene is located (Case 9). Dual-color FISH showed tumor cells with co-localization of amplified *ERBB2* (orange) and the corresponding amplified centromeric 17 lesion on which it is located (green). Large yellow signals overlap orange and green signals. The same field was observed using a tripleband filter (A), a SpectrumOrange^{TM}-specific filter (B) and a SpectrumGreen ^{TM} -specific filter (C).

Suppl. Fig. 2

Co-amplification of *TOP2A* and *ERBB2* in gastric cancer (Case 12).

Dual-color FISH analysis of *ERBB2* (green fluorescence) and *TOP2A* (orange fluorescence). This analysis showed that both signals are clustered and that these signals overlap. The same field was observed using the tripleband filter (A), SpectrumOrange^{TM}-specific filter (B) and SpectrumGreen ^{TM} -specific filter (C).

References

1. Jemal A, Bray F, Center MM, *et al.* Global cancer statistics. CA Cancer J Clin 2011;61:69-90.

2. Japanese Research Society for Gastric Cancer. The general rules for the gastric canceer study in surgery and pathology: Part I-Clinical classification. Jpn J Surg 1981;11:127-139.

3. Nashimoto A, Akazawa K, Isobe Y, *et al.* Gastric cancer treated in 2002 in Japan: 2009 annual report of the JGCA nationwide registry. Gastric cancer 2013;16:1-27.

4. 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.

5. Lee J, Ou SH. Towards the goal of personalized medicine in gastric cancer--time to move beyond HER2 inhibition. Part II: Targeting gene mutations and gene amplifications and the angiogenesis pathway. Discov Med 2013; 16:7-14.

6. 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;29:4803-4810.

7. An X, Wang F, Shao Q, *et al.* MET amplification is not rare and predicts unfavorable clinical outcomes in patients with recurrent/metastatic gastric cancer after chemotherapy. Cancer 2014;120:675-82.

8. Hara T, Ooi A, Kobayashi M, *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.

9. Jung EJ, Jung EJ, Min SY, Kim MA, Kim WH. Fibroblast growth factor receptor 2 gene amplification status and its clinicopathologic significance in gastric carcinoma. Hum Pathol 2012;43:1559-1566.

10. Matsumoto K, Arao T, Hamaguchi T, *et al.* FGFR2 gene amplification and clinicopathological features in gastric cancer. Br J Cancer 2012;106:727-732.

11. Smolen GA, Sordella R, Muir B, *et al.* Amplification of MET may identify a subset of cancers with extreme sensitivity to the selective tyrosine kinase inhibitor PHA-665752. Proc Natl Acad Sci USA 2006; 103:2316-21.

12. Xie L, Su X, Zhang L, *et al.* FGFR2 gene amplification in gastric cancer predicts sensitivity to the selective FGFR inhibitor AZD4547. Clin Cancer Res 2013; 19:2572-2583.

13. 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;1:73-684.

14. Liu YJ, Shen D, Yin X, *et al.* HER2, MET and FGFR2 oncogenic driver alterations define distinct molecular segments for targeted therapies in gastric carcinoma. Br J Cancer 2014;110:1169-1178.

15. Lee HE, Kim MA, Lee HS, *et al.* MET in gastric carcinomas: comparison between protein expression and gene copy number and impact on clinical outcome. Br J Cancer 2012;107:325-333

16. Tajiri R, Ooi A, Fujimura T, *et al.* Intratumoral heterogeneous amplification of ERBB2 and subclonal genetic diversity in gastric cancers revealed by multiple ligation-dependent probe amplification and fluorescence in situ hybridization. Hum Pathol 2014; 45:725-734.

17. 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.

18. Flemming ID, Cooper IS, Henson DE, et al (eds). Stomach. In: AJCC Cancer Staging Manual, 5th edn, ch. 10. Lippincott Williams and Wilkins: Philadelphia, 1997, pp71-76.

19. Fenoglio-Preiser C. Tumours of the stomach. In: Hamilton S, Aaltonen, SA (eds). WHO classification od tumours, IARC Press: Lyon, 2000, pp 37-52.

20. Takehana T, Kunitomo K, Suzuki S, *et al.* Expression of epidermal growth factor receptor in gastric carcinomas. Clin Gastroenterol Hepatol 2003; 1:438-445.

21. Rohmann E, Brunner HG, Kayserili H, *et al.* Mutations in different components of FGF signaling in LADD syndrome. Nature Genet ayserili H, et al. Mutations in different components of FGF signaling in LADD syndrome. Nature Genet 2006; 38:414-417.

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; 25:118-145.

23. 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; 33:21-28.

24. Starczynski J, Atkey N, Connelly Y, *et al.* HER2 gene amplification in breast cancer: a rogues' gallery of challenging diagnostic cases: UKNEQAS interpretation guidelines and research recommendations. Am J Clin Pathol 2012;137:595-605.

25. Mitsui F, Dobashi Y, Imoto I, *et al.* Non-incidental coamplification of Myc and ERBB2, and Myc and EGFR, in gastric adenocarcinomas. Mod Pathol 2007;20:622-631.

26. Kim C. Trastuzumab sensitivity of breast cancer with co-amplification of HER2 and cMyc in vivo. Breast Cancer Res Treat 2005; 54(Suppl 1):S6 (abstract).

27. Perez EA, Jenkins RB, Dueck AC, *et al.* C-MYC alterations and association with patient outcome in early-stage HER2-positive breast cancer from the north central cancer treatment group N9831 adjuvant trastuzumab trial. J Clin Oncol 2011; 29:651-659.

28. Nair R, Roden DL, Teo WS, *et al.* c-Myc and Her2 cooperate to drive a stem-like phenotype with poor prognosis in breast cancer. Oncogene 2013;33:3992-4002.

29. 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; 8:1107-1116.

30. Noel EE, Yeste-Velasco M, Mao X, *et al.* The association of CCND1 overexpression and cisplatin resistance in testicular germ cell tumors and other cancers. Am J Pathol 2010; 176:2607-2615.

31. Spencer-Dene B, Sala FG, Bellusci S, *et al.* Stomach development is dependent on fibroblast growth factor 10/fibroblast growth factor receptor 2b-mediated signaling. Gastroenterology 2006; 130:1233-1244.

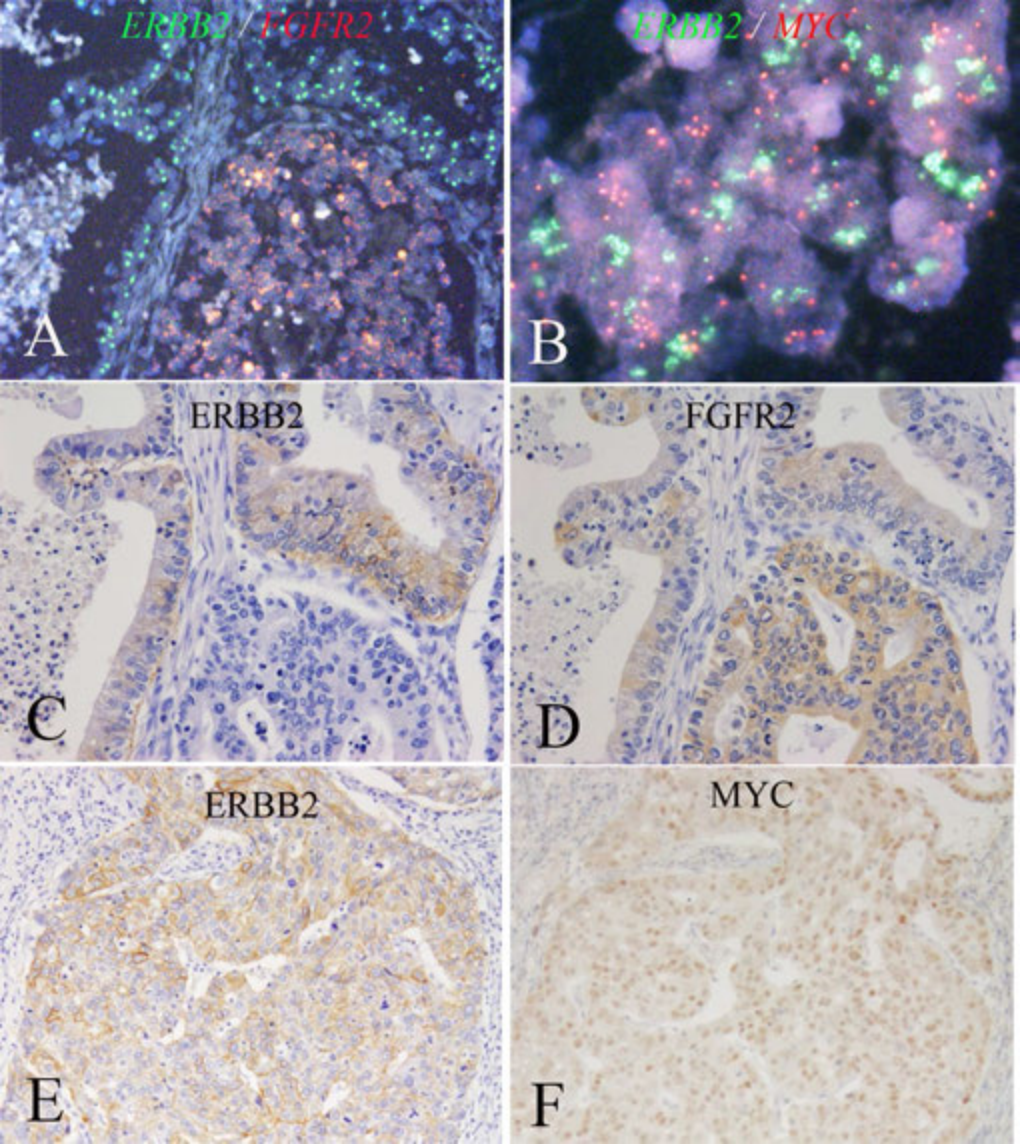
32. McInnes C, Wang S, Anderson S, *et al.* Structural determinants of CDK4 inhibition and design of selective ATP competitive inhibitors. Chem Biol 2004;11:525-534.

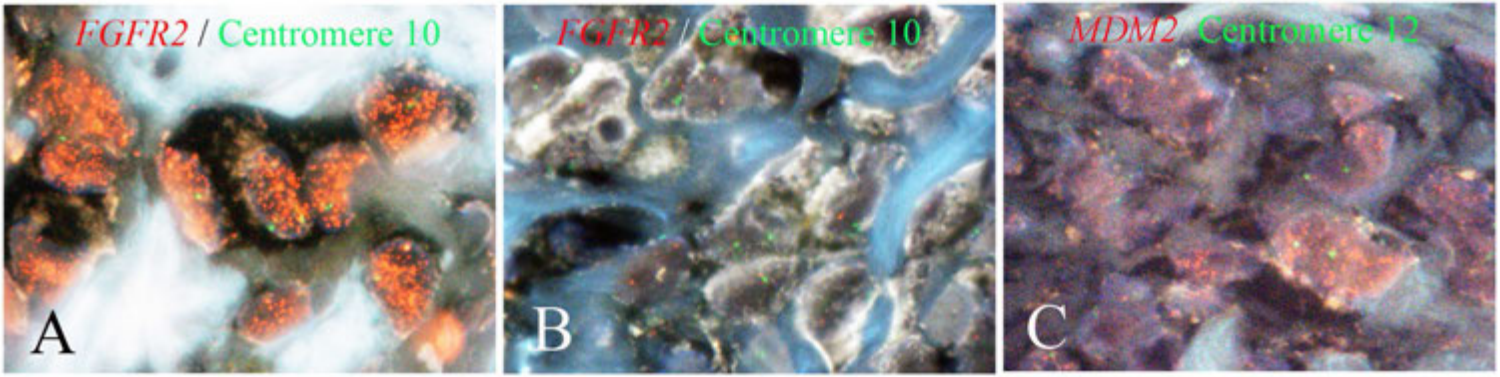
33. Vassilev LT, Vu BT, Graves B, *et al.* In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004; 303:844-848.

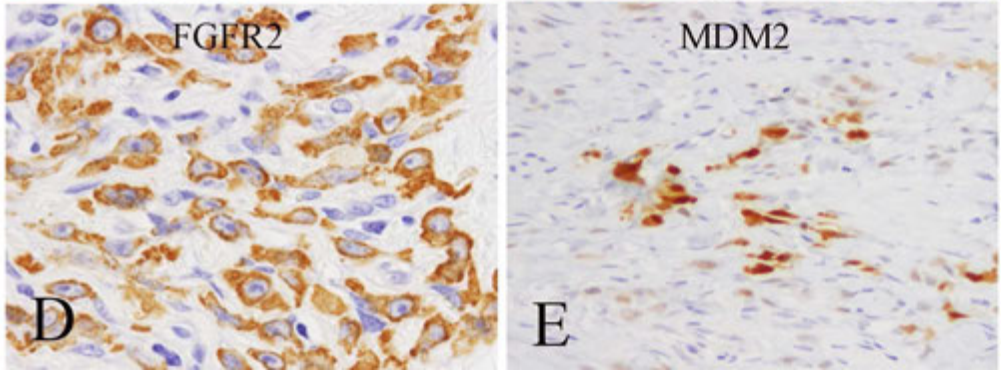
34. Muller CR, Paulsen EB, Noordhuis P, *et al.* Potential for treatment of

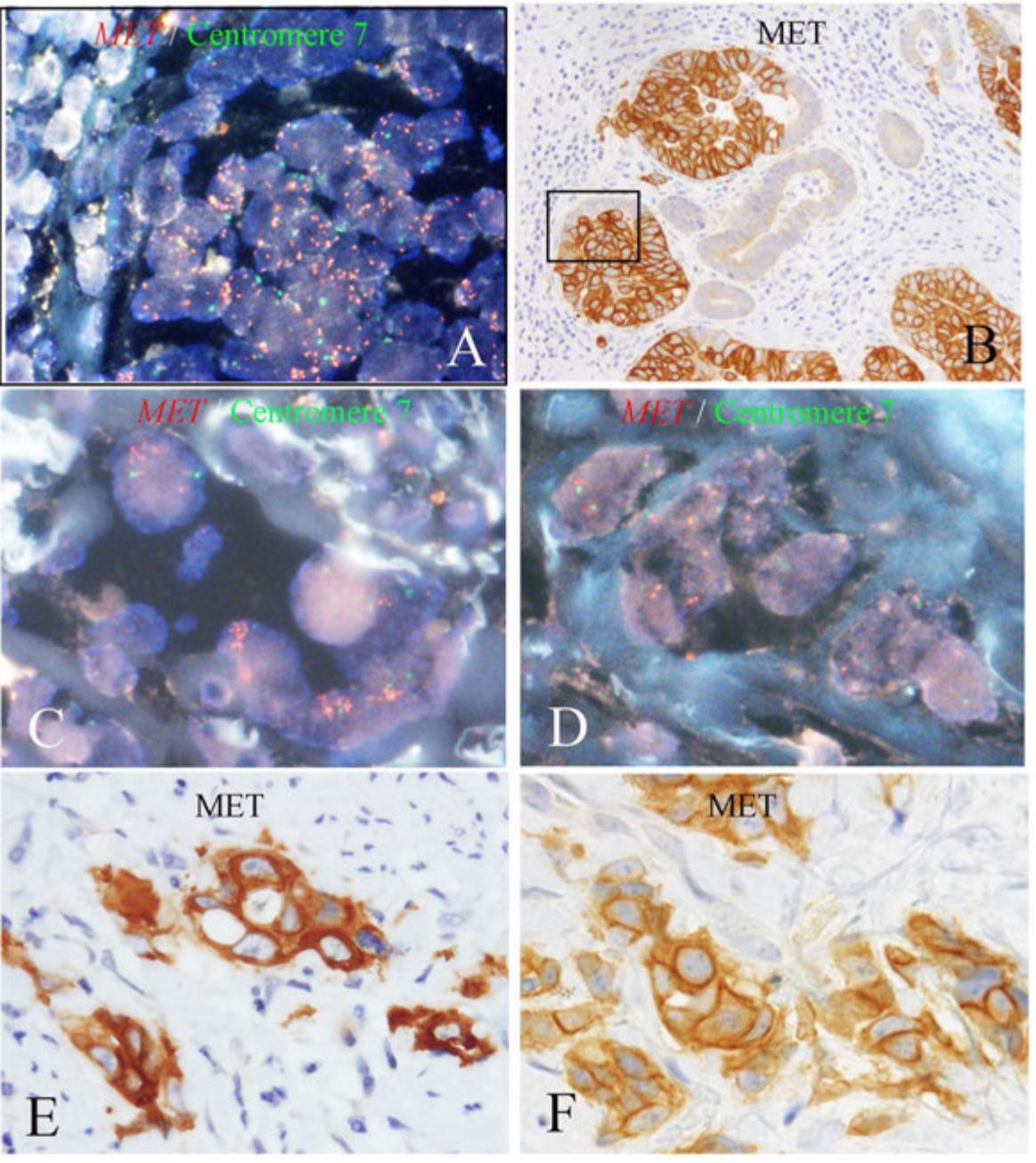
liposarcomas with the MDM2 antagonist Nutlin-3A. Int J Cancer 2007; 121:199-205.

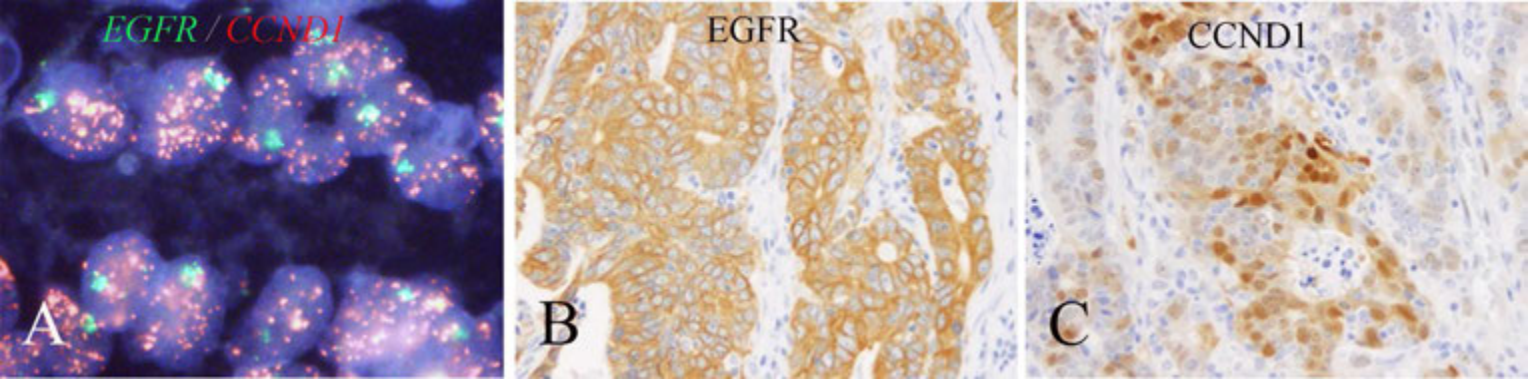
35. Moelans CB, de Weger RA, van Diest PJ. Multiplex ligation-dependent probe amplification to detect HER2 amplification in breast cancer: new insights in optimal cut-off value. Cell Oncol 2012;32:311-312.


ERBB2 / FGFR2
A
ERBB2 / MYC
B
ERBB2
C
FGFR2
D
ERBB2
E
MYC
F


FGFR2 / Centromere 10
A
FGFR2 / Centromere 10
B
MDM2 Centromere 12
C


FGFR2
D
MDM2
E


MET / Centromere 7
A
MET
B
MET / Centromere 7
C
MET / Centromere 7
D
MET
E
MET
F


EGFR / CCND1
A
EGFR
B
CCND1
C

Name of gene	*MDM4*	*FGF10*	*EGFR*	*MET*	*SMO*	*BRAF*	*FGFR1*	*MYC*	*FGFR2*	*CCND1*	*CDK4*	*MDM2*	*ERBB2*	*TOP2A*	*AURKA*	*AR*
Chromosomal locus of gen	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

Antibody	Clone	Dilution	Antigen retreval	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, Pleeasanton, CA, USA
CCND1	SP4	Prediluted	PC pH6, Citrate	Nichirei, Tokyo, Japan

Case No.	Histological 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	IIB							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