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Non-incidental co-amplification of *Myc* and *ERBB2*, and *Myc* and *EGFR*, in gastric adenocarcinomas.

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Abstract

This study was conducted to assess the frequencies of protein overexpression and gene amplification of *Myc* and to identify the mechanisms of *Myc* gene amplification, especially with regards to its possible co-amplification with *ERBB2* or *EGFR* in gastric adenocarcionomas. By immunohistochemical analysis of a total of 300 formalin-fixed and paraffin-embedded gastric adenocarcinomas, the nuclear overexpression of MYC was found in 47 tumors (16%). A fluorescence *in situ* hybridization analysis revealed that 9 (19%) of the 47 tumors with protein overexpression had cancer cells with high levels of *Myc* amplification, while only 7 (6%) of the 122 tumors without protein overexpression showed high-level *Myc* gene amplification. Such *Myc* amplification was significantly correlated with positive nuclear protein overexpression. The co-amplification of *ERBB2* or *EGFR* with *Myc* that was found in six and four cases, respectively, is believed to be non-incidental because those frequencies were significantly higher than the individual frequencies observed for the total examined cases (*ERBB2*: 7%; *EGFR*: 4%). The high levels of gene amplification of these three genes, as visualized by fluorescence *in situ* hybridization, could be broadly classified into two typical types, namely, “multiple scattered signals” and “large clustered signals”. Using two-color fluorescence *in situ* hybridization, the coexistence of co-amplified *Myc* and *ERBB2*, or *Myc* and *EGFR*, within single nuclei in various combinations of amplification types and copy numbers, could be ascertained in all nine cases, including one in which the synchronous “multiple scattered type” co-amplification of *Myc* and *ERBB2* was observed. In three tumors, co-amplification of *ERBB2* and *EGFR* was found; however, *ERBB2*- and *EGFR*-amplified cell populations were separate and mutually exclusive. We propose that the non-incidental co-amplification of *Myc* and either *ERBB2* or *EGFR* occurred through translocation and subsequent rearrangement.

Key words: *Myc*, *ERBB2*, *EGFR*, fluorescence *in situ* hybridization, gene amplification, gastric cancer

Introduction

The concept that multiple genetic alterations affecting protooncogenes and tumor suppressor genes are involved in the development of various human cancers is now widely accepted. However, the identification of the genes that are responsible for gastric cancer initiation and progression remains controversial. Thus far, genetic alterations previously reported in gastric carcinomas include the amplification of the *ERBB2*, *FGFR2*, *EGFR*, *MET* and *Myc* genes and point/frame shift mutations of the *KRAS*, *TP53*, *APC* and mismatch repair genes (1-5).

The *Myc* located on 8q24 encodes a transcription factor that is likely to contribute to tumorigenesis via its up-regulation, which would result in unrestrained cellular proliferation, blocking of differentiation, and promotion of genomic instability, including gene amplification, karyotypic abnormality, and hypersensitivity to DNA-damaging agents (6, 7). A number of alterations, including gene amplification, chromosomal translocation, insertional mutations, altered transcriptional elongation rates, and a prolonged mRNA half-life (7), affect MYC expression in various neoplasms. Gastric carcinomas studies using Southern blot (8-10) or comparative genetic hybridization (CGH) (11) revealed that *Myc* was amplified in a small fraction of gastric cancers. However, there have been very few comprehensive studies simultaneously examining protein expression and gene amplification of *Myc* in gastric cancer.

ERBB2 and *EGFR*, which are located at chromosome band 17q12-q21 and 7p12, encode the 185 kD and 170 kD plasma membrane glycoproteins, respectively. They are members of the family of tyrosine kinase growth factor receptors (TKGFR), share an approximately 50% overall homology, and are composed of an N-terminus extracellular ligand-binding domain, a transmembrane lipophilic segment, and a C-terminus intracellular region containing a tyrosine kinase domain (12). Stimuli through these receptors, such as those initiated by high-affinity ligand binding, activate a cascade of biochemical and physiological responses that are relayed to transcription factors, resulting in changes in gene and protein expression. In our previous studies combining immunohistochemistry and fluorescence *in situ* hybridization (FISH), we demonstrated that ERBB2 and EGFR are overexpressed in 8-10% (4, 13), and 10% (3), respectively, of gastic cancers, due primarily to gene amplification.

Co-amplification of *Myc* and *ERBB2* has been intensively investigated in breast cancers. In a FISH study on microarrays of more than a thousand breast cancers (14), co-amplification of *ERBB2* and *Myc* were found at a frequency of 2.4%. This was 2.6 times higher than expected based on the individual frequencies, with a worse prognosis

than tumors having only one of these amplifications. In gastric cancer, similar to breast cancer, co-amplifications of *Myc* and *ERBB2* (8), as well as *Myc* and genes encoding other GFRTK family members such as *EGFR* (15), *FGFR2* (16)·(8) and *MET* (17), have been reported in sporadic clinical cases or cell lines. However, it remains to be elucidated whether (1) the co-amplification of the two non-syntenic genes observed in previous studies occurred incidentally or not, (2) whether it occurred in single cells in a tumor or not, and (3) if they are co-amplified in single cells, whether the co-amplified genes are located on single amplicons or not. FISH has the potential to answer these questions because it can reveal both gene amplification on a cell-by-cell basis and the intranuclear topology of two amplified genes (18).

This study was conducted to assess the exact frequencies of *Myc* amplification and to clarify the mechanisms of gene amplification of *Myc*, especially the possible simultaneous amplification with *ERBB2* or *EGFR* in gastric cancers.

Materials and Methods

Patients

A total of 300 patients with gastric adenocarcinoma who underwent surgery at the Department of Surgery, Yamanashi Medical University between 1998-2005 were examined. The clinicopathological data are summarized in **Table 1.** Cancer staging was done according to the TNM cancer staging system of the American Joint Committee of Cancer (19). The World Health Organization Classification of Tumors (20) was used to determine histological classification. This laboratory study was approved by the Institutional Review Board at the University of Yamanashi, and written informed consent was obtained from all patients.

Serial sections cut from representative formalin-fixed, paraffin-embedded cancer tissues and placed on MAS^{TM}-coated slides (Matsunami Ltd., Tokyo, Japan) were used for hematoxylin-eosin staining, immunohistochemical detection, and a FISH analysis.

Immunohistochemistry

The immunohistochemical detection of MYC, ERBB2 and EGFR proteins was carried out on all the primary tumors. Monoclonal antibody (9E10, Pharmingen, San Diego, CA; working dilution, 1:30) against human MYC, a polyclonal antibody against the internal domain of the human ERBB2 protein (Nichirei, Tokyo, Japan; working dilution, 1:100), and a monoclonal antibody against the external domain of human EGFR (Novocastra Lab, Newcastle, UK; working dilution, 1:20) were used. The

specificities and sensitivities of the antibodies against ERBB2 and EGFR have been previously verified (3-5). For the immunohistochemical detection of these proteins, a high-temperature antigen unmasking technique was used. Antibody binding was visualized by the LSAB™ system (DakoCytomation, Kyoto, Japan). In each analysis of ERBB2 or EGFR, a gastric cancer section in which overexpression had been previously confirmed was included as a positive control.

The immunohistochemical analyses were reviewed by two examiners (FM, YD) who were unaware of the gene amplification data. For the evaluation of MYC staining, only nuclear immunostainings significantly higher than those of control cells of normal gastric mucosa were considered to be positive. For evaluation of ERBB2 and EGFR positivities, each tumor, or portion of tumor, was scored according to the criteria recommended by Dako (Glostruo, Denmark) for the HercepTest, except that the quantity of positive cells was not considered: no discernible staining, or background–type staining only; 1+, equivocal discontinuous membrane staining; 2+ unequivocal membrane staining with moderate intensity; 3+, strong and complete plasma membrane staining. Samples exhibiting 2+ or 3+ immunostaining were considered positive for overexpression.

FISH

A FISH analysis of *Myc* amplification was applied to all MYC-overexpressing primary tumors (47), as well as 122 non-overexpressing tumors selected at random. When primary tumors were amplification-positive, their metastatic lymph nodes, if any, were also examined for *Myc* amplification. Primary tumors with positive immunohistochemical staining (2+ and 3+) for either ERBB2 or EGFR were analyzed for *ERBB2/EGFR* amplification using FISH, since we previously demonstrated that only cases showing 2+ or 3+ positivity in immunohistochemistry, based on our criteria, were associated with gene amplifications of *ERBB2* and *EGFR* (3, 4, 21).

When *Myc*-amplification was positive, the primary tumors and their nodal metastases then underwent FISH examination for amplification of *ERBB2* and *EGFR*, even if the IHC result was negative. The tumors exhibiting a co-amplification of *Myc* and either *ERBB2* or *EGFR* were further examined, by simultaneous hybridization with two probes, for the co-existence of the amplified genes in single cells. For FISH probes, P1-artificial chromosome clone RP1-80K22, specific to *Myc*, and bacterial artificial chromosomes RP11-62N23 and RP11-339F13, specific to *ERBB2* and *EGFR*, respectively, were labeled with SpectrumOrange™ with a nick translation kit (Vysis, Downers Grove, IL). For the detection of gene amplification, a

SpectrumGreen™–labeled pericentromeric probe (Vysis), specific to each chromosome on which the particular gene was located, was co-hybridized in order to standardize the chromosome number. For simultaneous hybridization with *ERBB2* or *EGFR*, the *Myc* probe labeled with SpectrumGreen™ was used.

The removal of protein from the tissue sections was conducted as previously described (3, 4, 21). Denaturation, hybridization, and post-hybridization washing were carried out according to Vysis's protocol. The tissue sections were counterstained with DAPI II (Vysis) and examined with a fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Triple Bandpass Filter set (Vysis) for DAPI II, SpectrumOrange™ and SpectrumGreen™, and a filter set specific to SpectrumGreen™. As positive controls, gastric cancer tissue that had been previously confirmed to exhibit amplification of *ERBB2* or *EGFR* were used (3, 4). The FISH images were recorded through a cooled charged coupled device camera (DP-70) linked to a computer software program (DP manager, Olympus).

The copy numbers of each gene and centromere signals were counted, respectively, in at least 30 cancer nuclei and evaluated as follows. Basically, a cell in which the number of gene signals was greater than the number of centromeric signals was interpreted as positive for amplification. Among these, i) a definite cluster of gene signals or a total of 10 or more gene signals was scored as "high-level amplification", and ii) 3-9 signals in more than 60% of the tumor cells were "low-level amplification". In addition, iii) more than two signals accompanied by the identical number of centromere signals in more than 60% of the tumor cells were "polysomy" (3, 4), and iv) only two gene signals in more than 60% of the tumor cells were scored as "disomy". The extent (%) of such amplification of the cells was determined in a representative section from each tumor.

Statistics

The association between the amplification and overexpression of MYC, and any associations between the overexpression and amplification of *Myc*, and clinicopathological profiles of the tumors were analyzed for significance using either Fisher's exact probability test or the chi-square test. In addition, the association between the *Myc* amplification and either the *ERBB2 o*r *EGFR* amplification was determined by chi-square analysis.

Results

Myc

Overexpression of MYC protein was found in 47 (16%) of the 300 gastric carcinomas, although IHC results were equivocal in several tumors. (**Fig. 1A**.) FISH analysis revealed 16 tumors with *Myc* amplification (**Table 2**): 9 （15%） of the 47 tumors with protein overexpression had cancer cells with high-level amplification of *Myc*, although among the 122 tumors without protein over-expression only 7 (6%) had the gene amplification (three with high- and four with low-level amplification). The *Myc* amplification was significantly correlated with positive nuclear immunostaining (Fischer's exact probability test, P=0.011)

Overexpression of ERBB2 was found in 23 of the 300 tumors (8%) (**Fig. 1B**). Corresponding to the protein overexpression, high-level amplification of *ERBB2* was found in all cases except one, or 22 (7%) of the total 300 examined tumors overall. Overexpression of EGFR was found in 24 of the 300 tumors (**Fig. 1C**), and *EGFR* amplification was found in 12 of them (4%): 10 were high-level and 2 were low-level amplification.

The high-level gene amplification of *Myc*, *ERBB2*, and *EGFR*, as visualized by FISH, could be broadly classified into two typical types: multiple scattered signals ("MS") (**Fig. 2A**) and large clustered signals ("LC") (**Fig. 2B**), as reported in previous FISH studies (3, 4, 21, 22). The clinicopathological profiles and results of IHC and FISH for the 16 cases with *MYC* amplification are summarized in **Table 3.** Amplification pattern of *Myc* was found to be a combination of "MS" and "LC" in four cases (**Fig. 2C**). Among the total of 153 tumors without *MYC* amplification, 15 of 38 tumors with protein overexpression and 57 of 115 tumors without overexpression were polysomy 8, and the remaining 81 tumors were disomy 8. Statistically, no significant correlation was found between Polysomy 8 and MYC overexpression (Chi-square test)

ERBB2 and *Myc*

Amplification of *ERBB2* was found in 6 of 16 tumors with *Myc* amplification. In those tumors, the co-existence of amplified *ERBB2* and *Myc* in single cells was demonstrated. There were various combinations of amplification types between the two genes, as shown in **Fig. 3A-E** and as is summarized in **Table 3.** The cancer cells with co-amplification were major populations in two tumors (cases 9 and 10)**,** whereas in the other four cases (3, 6, 12 and 13) *ERBB2*–amplified cells occupied portions of the *Myc*-amplified tumor cells, but *Myc*-amplified cells without *ERBB2* amplification were also found **(Fig. 3B&C)** In five of six tumors (case 10 being the exception), the distribution and/or the signal numbers of *ERBB2* and *Myc* in individual nuclei were

different, and only occasionally doublets consisting of *ERBB2* and *Myc* were observed. However, for case 10, FISH showed that almost all the amplified signals of *Myc* and *ERBB2* overlapped, implicating that the number and distribution of *Myc* and ERBB2 were almost the same, as shown in **Figs. 3D&E**.

EGFR and *Myc*

Amplification of *EGFR* was found in 4 of 16 tumors with *MYC* amplification. In these four tumors co-amplification of *EGFR* and *MYC* in single nuclei was confirmed by dual-color FISH. Almost all the cancer cells from case 16 had co-amplified *Myc* and *EGFR* in MS (**Figs. 3F&G**); in the other cases EGFR amplification was found in a portion of *Myc*–amplified cells. In all cases, amplification patterns and/or number of copies of the two genes were dissimilar, as shown in Table 3.

ERBB2 and EGFR

Co-amplification of *ERBB2* and *EGFR* within a single tumor was found in three tumors, including case 6 which also had co-amplification of *Myc*. In these tumors, *ERBB2*- and *EGFR*-amplified cell populations were separate and mutually exclusive, and co-amplification in single cells was not detected.

Lymph nodes

Among the 16 cases exhibiting gene amplification of *Myc* in the primary tumors, 10 had lymph node metastases, and from 9 of the 10 cases the metastatic nodes were available for this study. When the gene amplification status of primary and metastatic tumors was compared, the concordant was found in three cases, as shown in Table 3. In case 13, *ERBB2*-amplified cells of “MS” type was the predominant population, although the primary tumor had *ERBB2*-amplified cells with “LC“ as a minor tumor component.

Statistics

The clinicopathological profiles of the cases with MYC overexpression and the cases with *Myc* amplification are compared with those of all examined cases in **Table 1**. MYC overexpression was frequently observed among the well/moderately differentiated tubular adenocarcinomas, however, no association was seen between the gene amplification of *Myc* and the histology of the tumors or the clinical stages.

The frequencies of gene amplification of *ERBB2* and *EGFR* were 7% (22 tumors) and 4% (12 tumors) out of the total of 300 tumors, respectively. Based on these

individual frequencies, the present rates of occurrence of *ERBB2* amplification (6 of 16) and *EGFR* amplification (4 of 16) among the cases with *Myc* amplification were highly statistically significantly (chi square: p=0.00019 and 0.0017, respectively).

Co-amplification of *ERBB2* and *EGFR*, found in three tumors, showed a marginal trend but was not statistically significant (Fischer's direct probability test, p=0.08).

Discussion

Gene amplification is one of the important genetic changes found in cancer cells. In the previous studies using Southern blot or CGH (8-11), the frequency of *Myc* amplification in gastric cancers ranged from 4% to 7.2%, and *ERBB2* amplification from 2.0% to 8.9%. The amplification frequencies we estimated for *Myc* (5%) and *ERBB2* (7%) in the present FISH study were comparable to those obtained by other authors. IHC detection of nuclear MYC significantly correlated with *Myc* amplification, but unlike that of ERBB2, a clear concordance of protein overexpression and gene amplification on a cell-by-cell basis was not observed. The discrepancy between the percentage with MYC overexpression and *Myc* amplification implies that increased expression is only partially due to gene amplification, and other mechanisms such as altered transcription rate or post-transcriptional modification may be responsible for sustained MYC overexpression. Another possible explanation is that MYC has a short half-life (20-30 min) (23). In addition, immunohistochemical analysis may be influenced by the amount of time taken between sampling and fixation, the length and method of fixation, and the degree of antigen retrieval.

In the present study, not only the non-incidental co-amplification of *ERBB2* or *EGFR* with *Myc* occurred in gastric cancers, but also the co-localization of the co-amplified genes in single nuclei was demonstrated. Although FISH on a tissue section has limited resolving power, it demonstrated the clonal heterogeneity of the gene amplification within single tumors and the intranuclear topologies of the amplified genes. This enables us to put forward several ideas about the cytogenetic mechanism of gene amplification, especially co-amplification of nonsyntenic oncogenes.

First, it is known that in mammalian cells high-level amplification is detected in two forms on the metaphase structures: homogeneously staining regions (HSR) and centromere-free extrachromosomal double minutes chromosomes (DM) (24). In addition, it is also known that in FISH on interphase nuclei, the amplified gene of interest in HSR appeared as one or two large clusters of signals (LC), while those in DMs appeared as multiple scattered signals (MS) (5, 25). As a result, the "MS" and

“LC” signals found in this study mostly, although not exclusively, correspond to the amplified genes in DM and HSR, respectively. Although the mechanisms of gene amplification and generation of DM and HSR in tumor cells are still somewhat obscure, at least two different initial gene amplification mechanisms are known: HSR is considered to be formed by breakage-fusion-bridge cycles (BFB) while DMs are explained by looping out (LO) of extra-chromosomal sequences. DMs can also be formed in HSR breakdown (24, 25) and they also relocate in a genome to form HSRs (26). Concerning MYC, it has been shown that amplified *Myc* in DMs, seen in direct tumor cell preparations from a colon cancer or a leukemia in later passage, were found intrachromosomally integrated within an HSR or an abnormally banded region (27, 28). On the other hand, in a murine pancreatic tumor cell line TD2, amplified *Myc* exists in the transition between being present in the form of DMs or a single HSR in chromosome 6. Interestingly, these DMs were demonstrated to have a minute fragment from chromosomes 6 near the *Myc*. This finding raises the possibility that these DMs derived from the HSR on chromosome 6 (29). In the present study, as shown in Table 3, *Myc* amplification of “HSR” and “DM” types were found to be mixed in four cancers. This fact supports the hypotheses that both types of amplification seem to be closely related and that amplicons can change their forms in clinical tumors, similar to that observed in cultured cells. The clinical significance of these different amplification patterns remains to be clarified; however, one study using a human tumor cell line demonstrated that in response to chemotherapy, amplified *Myc* located on DMs decreased, correlating with a dramatic reduction in tumorigenesis in nude mice. In contrast to this, the same therapy had no effect on gene copy number or tumorigenesis in a closely related cell line containing the same number of intra-chromosomally amplified *Myc* genes (30).

One of the cellular functions implicated by the action of MYC is destabilization of the cellular genome, (6, 31) which could facilitate the accumulation of subsequent genetic or chromosomal aberrations. In cases 3, 7, 12, 13, and 15, *Myc* amplification-positive populations contained both amplified and non-amplified subpopulations of *ERBB2* or *EGFR*. These findings suggest that the amplification of *ERBB2* or *EGFR* occurred after the *Myc* amplification in those particular tumors. A study using cell lines of various cell type and species showed that elevated levels of MYC protein affect subsequent gene amplification in a non-random manner (31). Among the hematopoietic tumors induced in transgenic mouse by the conditional expression of MYC, the chromosomal gain of chromosome 11, or of HSR loci on chromosome 11, were the frequent chromosomal changes (32). Although the amplified

genes were not specified in that study, mouse *ERBB2* and *EGFR*, which are located on chromosome 11, (33) may be involved in these lesions.

In cases 9, 10, and 16, the co-amplified cells consisted as a single major population, although the amplification types and copy numbers of co-amplified genes were different in cases 9 and 16. On simple theoretical grounds, amplification of the two genes in a large population would occur more easily through the propagation of a progenitor cell with co-amplified genes than by two-step amplification. Along this line, case 10, in which the synchronous amplification of *Myc* and *ERBB2* was found in almost all amplification-positive cells in the primary and metastatic tumors, is noteworthy. The overlapped FISH signals means the two genes are very close together, and probably are located on the same amplicons. A possible mechanism of the co-amplification deduced from this case is that the chromosomal fragment constituting both genes is amplified after both genes come close together by gene translocation, as shown in Fig. 4. Because two-color FISH is a sensitive and rapid means of identifying such a translocation, use of this technique in several *in vitro* studies showed that originally nonsyntecal genes in fact amplified in DMs or HSRs: some alveolar rhabdomyosarcomas contained a fusion of *PAX3* (2q35), or *PAX7* (1q36), and *FKHR* (13q14) in DMs (34); *Myc* and *ATBFI* (16q22) genes were found to be amplified on DMs in the neuroblastoma cell line SNB12; *CCND1*(11q13) and *FGFR1* (8p12) were co-amplified on two HSRs in the breast cancer cell line MDA-MB-134. Similarly, clustered co-amplification of *CCND1* and *FGFR1* was demonstrated by dual-color FISH using touch smear specimens obtained from six clinical breast cancers, and furthermore, with treatment to extend chromatin the overlapping clustered signals were demonstrated to be an arrangement of amplified genes (35). All the above results strongly suggest that the two genes come closely together by translocation before starting amplification by BFB or LO, as shown in Fig. 4.

The different amplification types (“HSR” type or “DM” type) and/or the different copy number of co-amplified genes in individual cancer cells that was observed in all cases except for case 10 may be explained by differences in the distance between the two translocated genes. For case 10, the genes were so closely located that they were not segregated as a result of chromosomal rearrangement. In the other cases, however, the two genes may be translocated closely but separately enough to produce variations in the amplification type and/or different copy number of genes following chromosomal rearrangement, as shown in Fig.4. Actually, *ERBB2* and *TOP2A*, which reside very close to each other at chromosome 17q12 (36) and are separated by approximately 690kb (NCBI human genome data base), are occasionally co-amplified in breast and

gastric cancers. Similarly, *KIT*, *VEGFR2* and *PDGFR*, originally located adjacently within a range of approximately 360Kb on chromosome 4q11-13, are co-amplified in some glioblastomas (37). However, in either tumors, dual-color FISH images of co-amplified genes were not simply overlapped signals or repetitive signal doublets (18, 37, 38). At the present time, it is most likely that *Myc* and *ERBB2*, or *Myc* and *EGFR*, amplification occurs simultaneously after translocation, although whether or not this is the only cytogenetic mechanism operating still remains to be elucidated.

Cancer progression occurs in a process in which different clones or subclones are produced by genetic instability and at the same time subject to selective forces. Thus, co-amplification of *Myc* and either *ERBB2* or *EGFR* may be a kind of collaboration that produces a growth advantage, although we could not find any clinical significance resulting from such co-amplifications. For example, *Myc* and *KRAS* synergistically function to induce the full malignant transformation of normal cells *in vitro* (39). The Ras/Raf/mitogen-activated protein kinase is one of the signaling cascades triggered by TKGFR activation. Thus *EGFR* or *ERBB2*, both members of TKGFR, and *Myc* may synergistically function in the development, maintenance and/or progression of gastric cancers. In contrast, both *EGFR* and *ERBB2* were found amplified in single tumors, but in mutually exclusive cancer cells, in three cases in the present study. This observation could be explained by ERBB2 and EGFR sharing the same downstream signal transduction systems, thus action of the two molecules might be functionally redundant. Recently, monoclonal antibodies to ERBB2 (trastuzumab) and EGFR have been clinically used for the treatment of patients with breast or colon cancers (40, 41). Interestingly, while patients with breast cancer with co-amplification of *Myc* and *ERBB2* had worse outcomes when treated with chemotherapy alone, addition of trastuzumab reversed this trend. This is suggested to be due to cancer apoptosis, because pro-apoptotic function of dysregulated MYC counterbalanced by anti-apoptotic signals provided by amplified ERBB2 is set free by trastuzumab (42).

It conclusion, *Myc* amplification was found in 5% (16 of 300) gastric carcinoma and was significantly correlated with nuclear overexpression of MYC protein. Dual color FISH revealed that i) the co-amplification of *Myc* with *ERBB2* or *EGFR* occurred non-incidentally, ii) this co-amplification occurred in single cells, and iii) this co-amplification probably involved single amplicons, at least in the initial stages. The knowledge of genetic heterogeneity in gastric cancers obtained by FISH may be useful for determining the optimal treatment targets and therapeutic schedules.

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References

[1] Kountouras J, Zavos C, Chatzopoulos D. New concepts of molecular biology on gastric carcinogenesis. Hepatogastroenterology 2005;52:1305-1312.

[2] Koo SH, Kwon KC, Shin SY, Jeon YM, Park JW, Kim SH, et al. Genetic alterations of gastric cancer: comparative genomic hybridization and fluorescence In situ hybridization studies. Cancer Genet Cytogenet 2000;117:97-103.

[3] Takehana T, Kunitomo K, Suzuki S, Kono K, Fujii H, Matsumoto Y, et al. Expression of epidermal growth factor receptor in gastric carcinomas. Clin Gastroenterol Hepatol 2003;1:438-445.

[4] Takehana T, Kunitomo K, Kono K, Kitahara F, Iizuka H, Matsumoto Y, 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. Int J Cancer 2002;98:833-837.

[5] Hara T, Ooi A, Kobayashi M, Mai M, Yanagihara K, Nakanishi I. Amplification of c-myc, K-sam, and c-met in gastric cancers: detection by fluorescence in situ hybridization. Lab Invest 1998;78:1143-1153.

[6] Felsher DW, Bishop JM. Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc Natl Acad Sci U S A 1999;96:3940-3944.

[7] Marcu KB, Bossone SA, Patel AJ. myc function and regulation. Annu Rev Biochem 1992;61:809-860.

[8] Mor O, Ranzani GN, Ravia Y, Rotman G, Gutman M, Manor A, et al. DNA amplification in human gastric carcinomas. Cancer Genet Cytogenet 1993;65:111-114.

[9] Ranzani GN, Pellegata NS, Previdere C, Saragoni A, Vio A, Maltoni M, et al. Heterogeneous protooncogene amplification correlates with tumor progression and presence of metastases in gastric cancer patients. Cancer Res 1990;50:7811-7814.

[10] Amadori D, Maltoni M, Volpi A, Nanni O, Scarpi E, Renault B, et al. Gene amplification and proliferative kinetics in relation to prognosis of patients with gastric carcinoma. Cancer 1997;79:226-232.

[11] Sakakura C, Mori T, Sakabe T, Ariyama Y, Shinomiya T, Date K, et al. Gains, losses, and amplifications of genomic materials in primary gastric cancers analyzed by comparative genomic hybridization. Genes Chromosomes Cancer 1999;24:299-305.

[12] Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyajima N, et al. Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor. Nature 1986;319:230-234.
[13] Ooi A, Kobayashi M, Mai M, Nakanishi I. Amplification of c-erbB-2 in gastric cancer: detection in formalin-fixed, paraffin-embedded tissue by fluorescence in situ hybridization. Lab Invest 1998;78:345-351.
[14] Al-Kuraya K, Schraml P, Torhorst J, Tapia C, Zaharieva B, Novotny H, et al. Prognostic relevance of gene amplifications and coamplifications in breast cancer. Cancer Res 2004;64:8534-8540.
[15] Nomura N, Yamamoto T, Toyoshima K, Ohami H, Akimaru K, Sasaki S, et al. DNA amplification of the c-myc and c-erbB-1 genes in a human stomach cancer. Jpn J Cancer Res 1986;77:1188-1192.
[16] Yanagihara K, Seyama T, Tsumuraya M, Kamada N, Yokoro K. Establishment and characterization of human signet ring cell gastric carcinoma cell lines with amplification of the c-myc oncogene. Cancer Res 1991;51:381-386.
[17] Seruca R, Suijkerbuijk RF, Gartner F, Criado B, Veiga I, Olde-Weghuis D, et al. Increasing levels of MYC and MET co-amplification during tumor progression of a case of gastric cancer. Cancer Genet Cytogenet 1995;82:140-145.
[18] 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;37:1333-1343.
[19] Flemming ICJ, Henson D, Hutter R, Kennedy B, Burphy G, o'Sullivan B, Sobin L, Yarbo J. AJCC cancer staging manual. 5th ed. Philadelphia: Lippincott Williams and Wilkins, 1997.71-76
[20] Fenoglio-Preiser C. Tumours of the stomach. In: Hamilton S, Aaltonen, LA, editor. WHO classification od tumours. Lyon: IARC Press, 2000.
[21] Ooi A, Takehana T, Li X, Suzuki S, Kunitomo K, Iino H, et al. Protein overexpression and gene amplification of HER-2 and EGFR in colorectal cancers: an immunohistochemical and fluorescent in situ hybridization study. Mod Pathol 2004;17:895-904.
[22] Nakazawa K, Dobashi Y, Suzuki S, Fujii H, Takeda Y, Ooi A. Amplification and overexpression of c-erbB-2, epidermal growth factor receptor, and c-met in biliary tract cancers. J Pathol 2005;206:356-365.
[23] Hann SR, Eisenman RN. Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells. Mol Cell Biol

1984;4:2486-2497.
[24] Cowell JK. Double minutes and homogeneously staining regions: gene amplification in mammalian cells. Annu Rev Genet 1982;16:21-59.
[25] Coquelle A, Rozier L, Dutrillaux B, Debatisse M. Induction of multiple double-strand breaks within an hsr by meganucleaseI-SceI expression or fragile site activation leads to formation of double minutes and other chromosomal rearrangements. Oncogene 2002;21:7671-7679.
[26] Myllykangas S, Knuutila S. Manifestation, mechanisms and mysteries of gene amplifications. Cancer Lett 2006;232:79-89.
[27] Alitalo K, Schwab M, Lin CC, Varmus HE, Bishop JM. Homogeneously staining chromosomal regions contain amplified copies of an abundantly expressed cellular oncogene (c-myc) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci U S A 1983;80:1707-1711.
[28] Nowell P, Finan J, Dalla-Favera R, Gallo RC, ar-Rushdi A, Romanczuk H, et al. Association of amplified oncogene c-myc with an abnormally banded chromosome 8 in a human leukaemia cell line. Nature 1983;306:494-497.
[29] Schreiner B, Greten FR, Baur DM, Fingerle AA, Zechner U, Bohm C, et al. Murine pancreatic tumor cell line TD2 bears the characteristic pattern of genetic changes with two independently amplified gene loci. Oncogene 2003;22:6802-6809.
[30] Von Hoff DD, McGill JR, Forseth BJ, Davidson KK, Bradley TP, Van Devanter DR, et al. Elimination of extrachromosomally amplified MYC genes from human tumor cells reduces their tumorigenicity. Proc Natl Acad Sci U S A 1992;89:8165-8169.
[31] Mai S, Hanley-Hyde J, Fluri M. c-Myc overexpression associated DHFR gene amplification in hamster, rat, mouse and human cell lines. Oncogene 1996;12:277-288.
[32] Karlsson A, Giuriato S, Tang F, Fung-Weier J, Levan G, Felsher DW. Genomically complex lymphomas undergo sustained tumor regression upon MYC inactivation unless they acquire novel chromosomal translocations. Blood 2003;101:2797-2803.
[33] Buchberg AM, Brownell E, Nagata S, Jenkins NA, Copeland NG. A comprehensive genetic map of murine chromosome 11 reveals extensive linkage conservation between mouse and human. Genetics 1989;122:153-161.
[34] Barr FG, Nauta LE, Davis RJ, Schafer BW, Nycum LM, Biegel JA. In vivo amplification of the PAX3-FKHR and PAX7-FKHR fusion genes in alveolar

rhabdomyosarcoma. Hum Mol Genet 1996;5:15-21.

[35] Bautista S, Theillet C. CCND1 and FGFR1 coamplification results in the colocalization of 11q13 and 8p12 sequences in breast tumor nuclei. Genes Chromosomes Cancer 1998;22:268-277.

[36] Jarvinen TA, Tanner M, Rantanen V, Barlund M, Borg A, Grenman S, et al. Amplification and deletion of topoisomerase IIalpha associate with ErbB-2 amplification and affect sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer. Am J Pathol 2000;156:839-847.

[37] Joensuu H, Puputti M, Sihto H, Tynninen O, Nupponen NN. Amplification of genes encoding KIT, PDGFRalpha and VEGFR2 receptor tyrosine kinases is frequent in glioblastoma multiforme. J Pathol 2005;207:224-231.

[38] Jarvinen TA, Tanner M, Barlund M, Borg A, Isola J. Characterization of topoisomerase II alpha gene amplification and deletion in breast cancer. Genes Chromosomes Cancer 1999;26:142-150.

[39] Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell 1987;49:465-475.

[40] Treish I, Schwartz R, Lindley C. Pharmacology and therapeutic use of trastuzumab in breast cancer. Am J Health Syst Pharm 2000;57:2063-2076; quiz 2077-2069.

[41] Moroni M, Veronese S, Benvenuti S, Marrapese G, Sartore-Bianchi A, Di Nicolantonio F, et al. Gene copy number for epidermal growth factor receptor (EGFR) and clinical response to antiEGFR treatment in colorectal cancer: a cohort study. Lancet Oncol 2005;6:279-286.

[42] .Kim C, Bryant J, Horne Z, Geyer CE Wickerham DL,Wolmark et al. Trastuzumab sensitivity of breast cancer with co-amplification of HER2 and cMyc in vivo. Breast Cancer Res Treat 2005;94(Suppl 1):S6 (abstract 46).

Figure legends

Figure 1

Results of the IHC. Cancer nuclei were immunostained for MYC (case9) (A). Cell membranes of intestinal type cancer cells were immunostained for ERBB2 (case 13) (B) and those of diffuse type cancer cells for EGFR (case 16) (C).

Figure 2

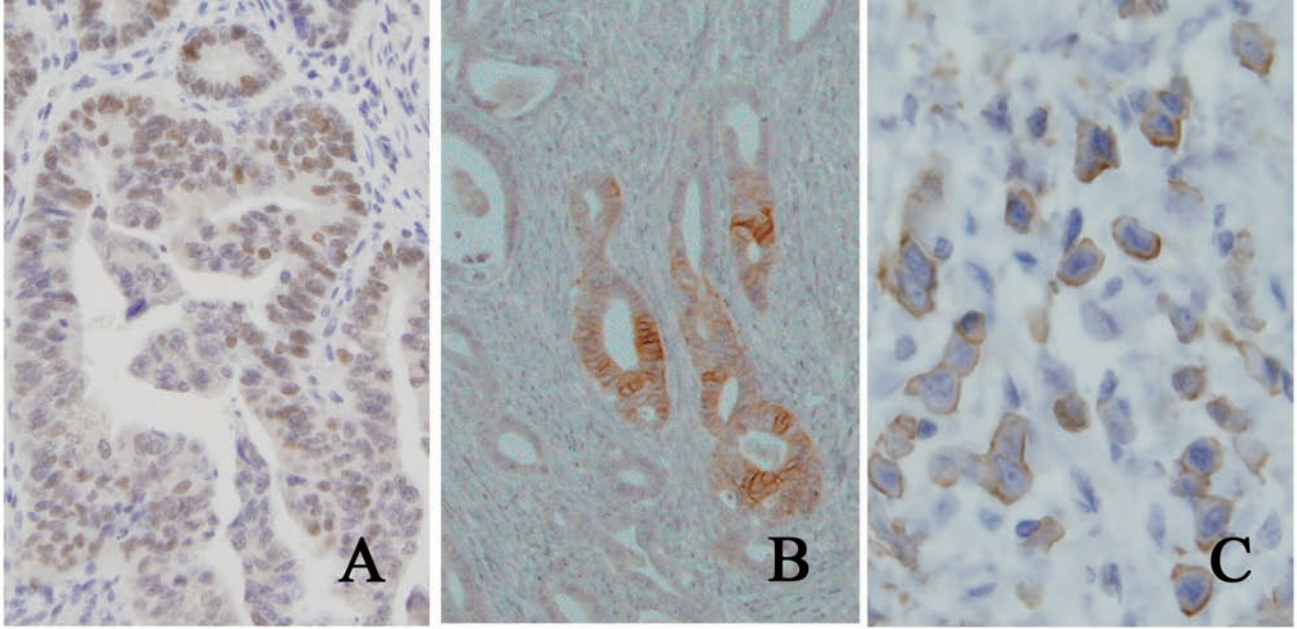
Dual-color FISH of *Myc* (orange signals) and centromere 8 (green signals).
Amplified *Myc* gene (orange) appears as multiple scattered signals "MS" (case 9) (A), or as large clusters "LC" (case 3) (B). The co-existence of cancer cells with both "MS" and "LC" (case 9) (C).

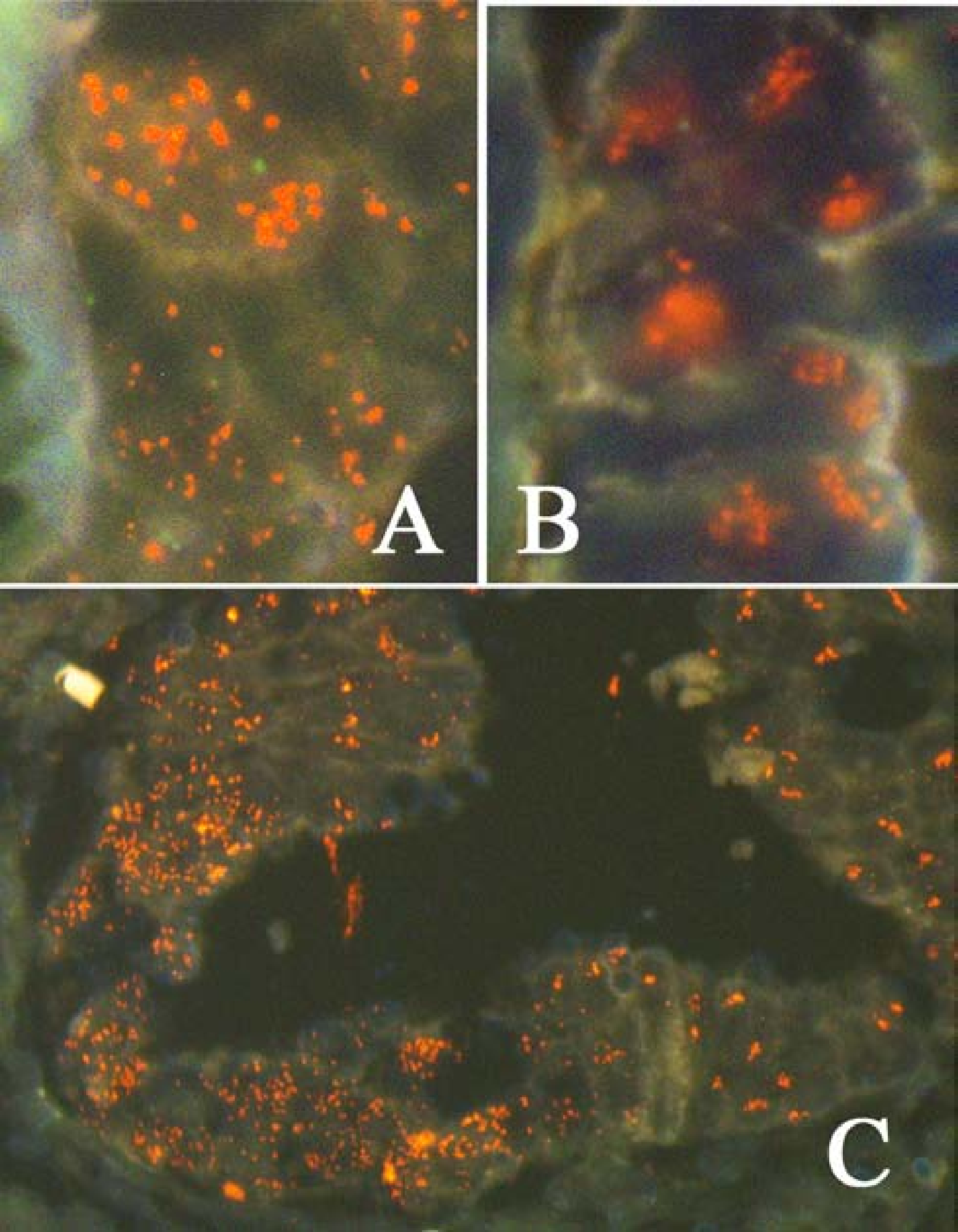
Fig 3

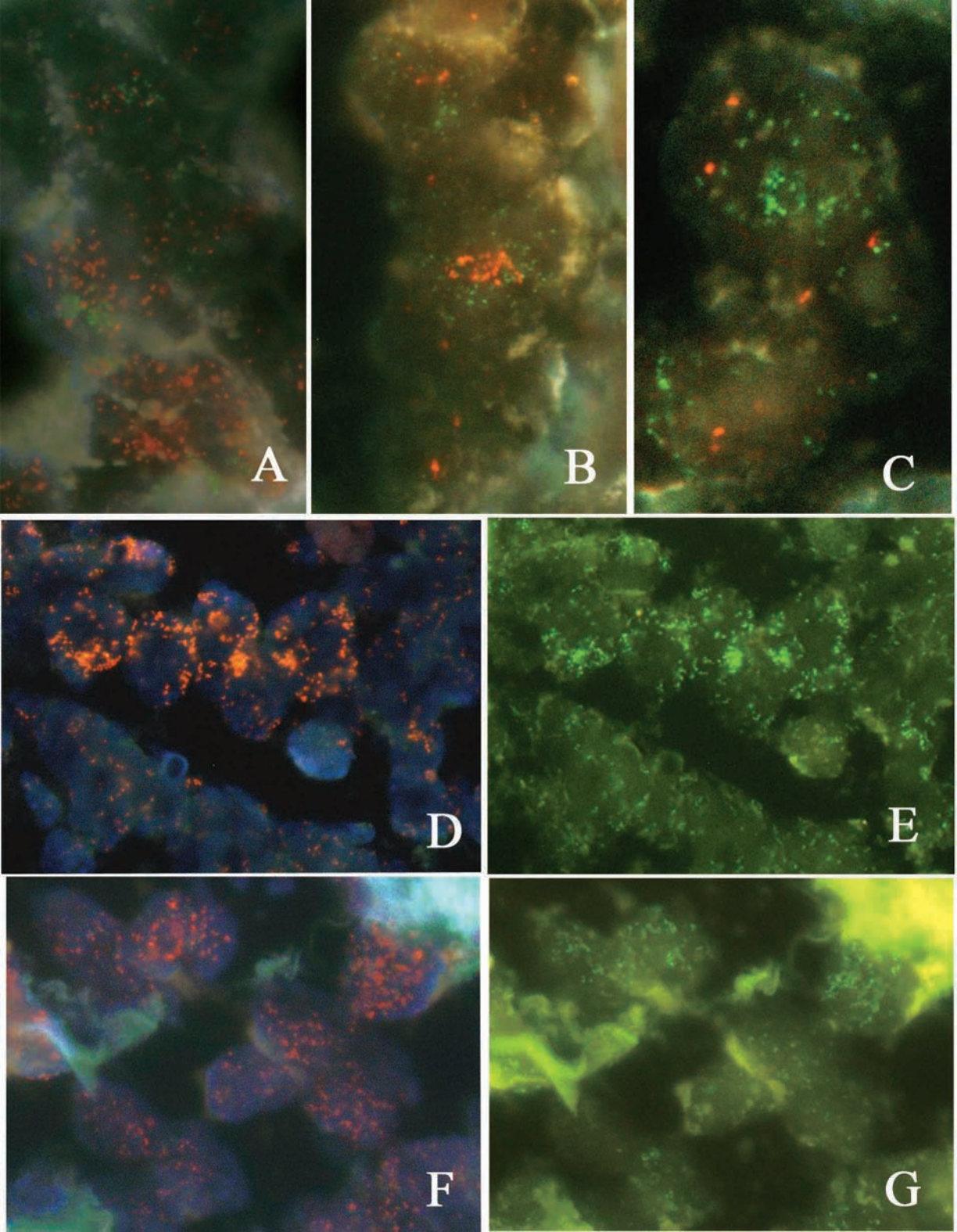
Dual color FISH of *ERBB2* (orange signals) and *Myc* (green signals) (A-E), and *EGFR* (orange signals) and *Myc* (green signals) (F&G). Co-amplified signals were found in single nuclei in various combinations of amplified patterns: *Myc* was amplified in LC and *ERBB2* in MS in case 3 (A), and *Myc* in MS and *EGFR* in LC in case 13 (B). Case 13 also had *Myc*-amplified cells without *ERBB2* amplification (C). In cases 10 and 16, through the triple-band filter *Myc* signals (green fluorescence) were not well discernible (D&F), but using a SpecrumGreen^{TM}-specific filter, the green fluorescence of the *Myc* gene was clearly observed (E&G). The number and distribution of *Myc* and *ERBB2* were almost the same in case 10 (D&E). In case 16, *EGFR* (orange signals) and *Myc* (green signals) were amplified in MS, however, differently distributed in the nuclei (F&G).

Figure 4

The proposed mechanisms leading to a co-amplification of *Myc* with either *ERBB2* or *EGFR.*


A
B
C


A
B
C


A
B
C
D
E
F
G

Speculated mechanism of the coamplification

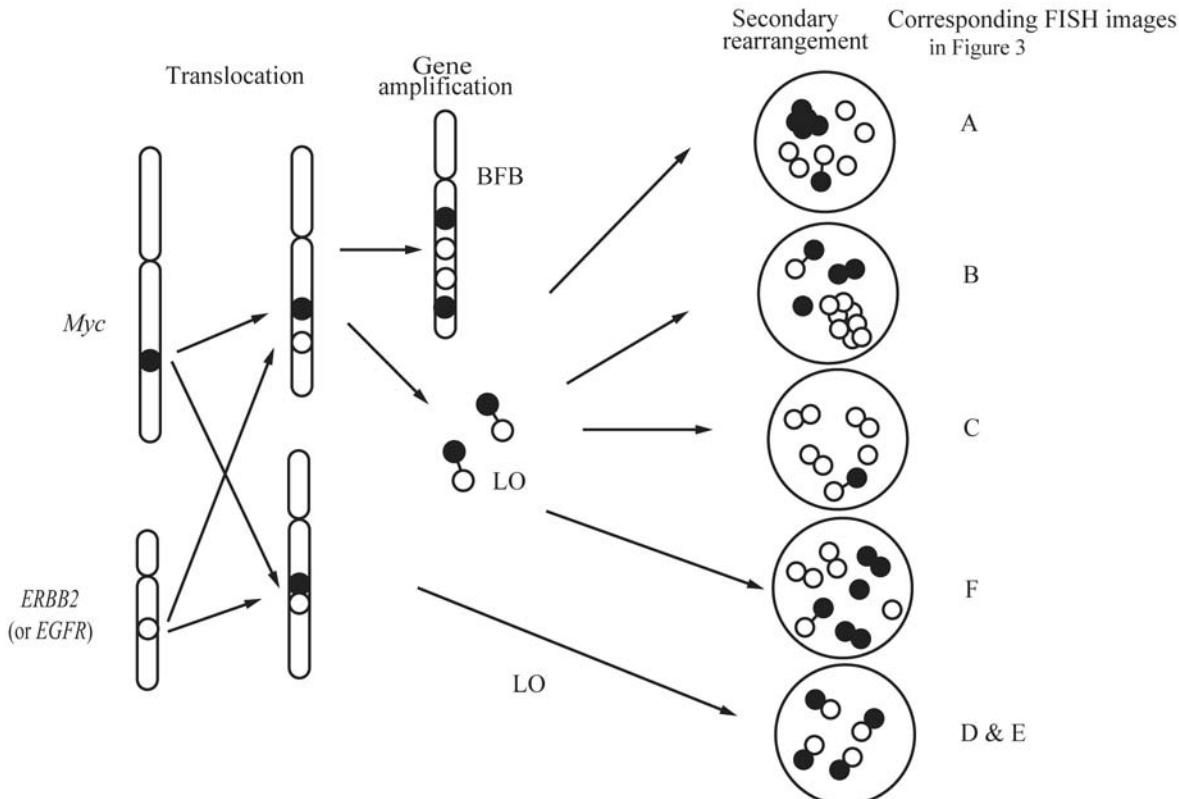


Table 1. Statistical Analysis

Variable	No. of tumors	Correlation with % of MYC overexpression	Correlation with % of *MYC* amplification
Histologic type			
wel	102	19	7
mod	69	26	7
por	81	9	4
muc	7	14	0
sig	41	5	2
P value		0.65[b)]	0.42[b)]
Stage			
IA	140	14	3
IB	42	12	5
II	31	29	7
IIIA	30	23	3
IIIB	20	10	15
IV	37	11	11
P value		0.20[b)]	0.16[b)]

[a)] Fisher's exact probability test
[b)] Chi-square for independent test

* $p<0.01$ ** $p<0.05$

Table 2 Correlation between protein overexpression (IHC) and gene amplification (FISH) of the *Myc*

	Nuclear overexpression	
	positive	negative
Gene amplification positive	9	7
negative	38	115

$p = 0.0076$

Table 3. Pathological Classification and Results of IHC and FISH Analysis of 16 Gastric Carcinomas with *Myc* Amplification

Case No.	Macroscopic classification	Histologic Classification	stage	*Myc*		*ERBB2*		*EGFR*		Amplification status of nodal metastases
				IHC	FISH (%[a])	IHC	FISH (%[a])	IHC	FISH (%[a])	
1	IIc	wel	IA	+	MS (90)	-	D	-	D	None
2	IIc	sig	IA	+	LC>MS (90)	-	D	-	D	None
3	IIb +IIa	mod	IA	+	LC>MS (60)	3+	MS (30)	1+	P	None
4	IIc	wel	IA	-	MS-LA(20)	-	D	-	P	None
5	2	mod	1B	-	LA(20)	-	D	-	D	None
6	3	mod	IB	-	LA (30)	2+	LC (30)	2+	LC (20)	None
7	IIc	mod	II	-	LA (30)	-	D	3+	MS (15)	*Myc* (LA) /*EGFR* (-)
8	2	wel	II	+	LC>MS (90)	-	P	1+	P	*Myc* (MS)
9	3	mod	IIIA	+	MS>LC (80)	3+	MS (80)	1+	D	*Myc* (MS) /*ERBB2* (MS)
10	2	mod	IIIA	+	MS (80)	3+	MS (80)	-	P	*Myc* (MS) /*ERBB2* (MS)
11	3	pap	IIIB	-	MS(20)	-	P	2+	P	*Myc* (MS)
12	3	mod	IIIB	-	LA (20)	3+	LC (5)	-	P	*Myc* (LA)/ *ERBB2* (LA)
13	3	mod	IIIB	+	MS (60)	3+	LC (5)	1+	D	*Myc* (−) / *ERBB2* (MS)
14	4	por	IV	-	MS(30)	-	P	-	D	*Myc* (−)
15	3	por	IV	+	MS (80)	-	P	2+	MS (10)	*Myc* (MS)/ *EGFR* (−)
16	3	por	IV	+	MS (90)	-	P	3+	MS (90)	Not obtained

%[a]: % of cancer cells with amplification

Abbreviations: wel, well-differentiated tubular adenocarcinoma; sig, signet-ring cell carcinoma; mod, moderately differentiated tubular adenocarcinoma; pap,papillary adenocarcinoma; por, poorly differentiated adencarcinoma; MS, multiple scattered signals;
LC, large clustered signals; LA, low grade amplification; D, disomy; P, polysomy; None, no nodal metastasis