Non-incident al coamplification of *Myc* and *ERBB2*, and *Myc* and *EGFR*, in gastric adenocarcinomas

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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 coamplification with *ERBB2* or *EGFR* in gastric adenocarcinomas. 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 (FISH) analysis revealed that nine (19%) of the 47 tumors with protein overexpression had cancer cells with high levels of *Myc* amplification, whereas only seven (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 coamplification of *ERBB2* or *EGFR* with *Myc* that was found in six and four cases, respectively, is believed to be non-incident al 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 FISH, could be broadly classified into two typical types, namely, 'multiple scattered signals' and 'large clustered signals'. Using two-color FISH, the coexistence of coamplified *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' coamplification of *Myc* and *ERBB2* was observed. In three tumors, coamplification of *ERBB2* and *EGFR* was found; however, *ERBB2*- and *EGFR*-amplified cell populations were separate and mutually exclusive. We propose that the non-incident al coamplification of *Myc* and either *ERBB2* or *EGFR* occurred through translocation and subsequent rearrangement.

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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 pre-

viously reported in gastric carcinomas include the amplification of the *ERBB2*, *FGFR2*, *EGFR*, *MET* and *Myc* genes and point/frameshift 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 upregulation, 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

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in various neoplasms. Gastric carcinoma 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 and 170 kDa 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 (IHC) and fluorescence *in situ* hybridization (FISH), we demonstrated that *ERBB2* and *EGFR* are overexpressed in 8–10%,^{4,13} and 10%,³ respectively, of gastric cancers, due primarily to gene amplification.

Coamplification 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 coamplification of *ERBB2* and *Myc* was 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, coamplifications 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 coamplification 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 coamplified in single cells, whether the coamplified 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 and 2005 were examined. The clinicopathological data are summarized in Table 1. Cancer staging was performed according to the TNM cancer staging system of the American Joint Committee of Cancer.¹⁹ The World Health Organization Classification of Tumors²⁰ was used to determine histological classification. This laboratory study was approved by the

Table 1 Statistical analysis

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

^aFisher's exact probability test.

^b χ^2 for independent test.

* $P < 0.01$, ** $P < 0.05$.

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 MASTM-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, USA; 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 LSABTM 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 (Glostrup, 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 47 MYC-overexpressing primary tumors, 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, as we previously demonstrated that only cases showing 2+ or 3+ positivity in IHC, 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 coamplification of *Myc* and either *ERBB2* or *EGFR* were further examined, by simultaneous hybridization with two probes, for the coexistence 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 SpectrumOrangeTM with a nick translation kit (Vysis, Downers Grove, IL, USA). For the detection of gene amplification, a SpectrumGreenTM-labeled pericentromeric probe (Vysis), specific to each chromosome on which the particular gene was located, was cohybridized in order to standardize the chromosome number. For simultaneous hybridization with *ERBB2* or *EGFR*, the *Myc* probe labeled with SpectrumGreenTM was used.

The removal of protein from the tissue sections was conducted as described previously.^{3,4,21} Denaturation, hybridization, and posthybridization 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, SpectrumOrangeTM and SpectrumGreenTM, and a filter set specific to SpectrumGreenTM. As positive controls, gastric cancer tissues 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.

Figure 1 Results of the IHC. Cancer nuclei were immunostained for MYC (case 9) (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).

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 χ^2 test. In addition, the association between the *Myc* amplification and either the *ERBB2* or *EGFR* amplification was determined by χ^2 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 (Figure 1a). FISH analysis revealed 16 tumors with *Myc* amplification (Table 2): nine (15%) of the 47 tumors with protein overexpression had cancer cells with high-level amplification of *Myc*, although among the 122 tumors without protein overexpression only seven (6%) had the gene amplification (three with high-level 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%) (Figure 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 (Figure 1c), and *EGFR* amplification was

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

$P=0.0076$.

found in 12 of them (4%): 10 were high-level and two 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') (Figure 2a) and large clustered signals ('LC') (Figure 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 (Figure 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 (χ^2 test).

ERBB2 and Myc

Amplification of *ERBB2* was found in six of 16 tumors with *Myc* amplification. In those tumors, the coexistence of amplified *ERBB2* and *Myc* in single

cells was demonstrated. There were various combinations of amplification types between the two genes, as shown in Figure 3a–e and as is

Figure 2 Dual-color FISH of *Myc* (orange signals) and centromere 8 (green signals). Amplified *Myc* gene (orange) appears as 'MS' (case 9) (a), or as large clusters 'LC' (case 3) (b). The coexistence of cancer cells with both 'MS' and 'LC' (case 9) (c).

summarized in Table 3. The cancer cells with coamplification 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 (Figure 3b and 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 Figure 3d and e.

EGFR and *Myc*

Amplification of *EGFR* was found in four of 16 tumors with *MYC* amplification. In these four tumors, coamplification of *EGFR* and *MYC* in single nuclei was confirmed by dual-color FISH. Almost all the cancer cells from case 16 had coamplified *Myc* and *EGFR* in MS (Figure 3f and 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*

Coamplification of *ERBB2* and *EGFR* within a single tumor was found in three tumors, including case 6 which also had coamplification of *Myc*. In these

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	<i>Myc</i>		<i>ERBB2</i>		<i>EGFR</i>		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)	<i>Myc</i> (LA)/ <i>EGFR</i> (–)
8	2	wel	II	+	LC>MS (90)	–	P	1+	P	<i>Myc</i> (MS)
9	3	mod	IIIA	+	MS>LC (80)	3+	MS (80)	1+	D	<i>Myc</i> (MS)/ <i>ERBB2</i> (MS)
10	2	mod	IIIA	+	MS (80)	3+	MS (80)	–	P	<i>Myc</i> (MS)/ <i>ERBB2</i> (MS)
11	3	pap	IIIB	–	MS (20)	–	P	2+	P	<i>Myc</i> (MS)
12	3	mod	IIIB	–	LA (20)	3+	LC (5)	–	P	<i>Myc</i> (LA)/ <i>ERBB2</i> (LA)
13	3	mod	IIIB	+	MS (60)	3+	LC (5)	1+	D	<i>Myc</i> (–)/ <i>ERBB2</i> (MS)
14	4	por	IV	–	MS (30)	–	P	–	D	<i>Myc</i> (–)
15	3	por	IV	+	MS (80)	–	P	2+	MS (10)	<i>Myc</i> (MS)/ <i>EGFR</i> (–)
16	3	por	IV	+	MS (90)	–	P	3+	MS (90)	Not obtained

%^a: % of cancer cells with amplification.

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

Figure 3 Dual-color FISH of *ERBB2* (orange signals) and *Myc* (green signals) (a–e), and *EGFR* (orange signals) and *Myc* (green signals) (f and g). Coamplified 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 and f), but using a SpectrumGreen™-specific filter, the green fluorescence of the *Myc* gene was clearly observed (e and g). The number and distribution of *Myc* and *ERBB2* were almost the same in case 10 (d and e). In case 16, *EGFR* (orange signals) and *Myc* (green signals) were amplified in MS, however, differently distributed in the nuclei (f and g).

tumors, *ERBB2*- and *EGFR*-amplified cell populations were separate and mutually exclusive, and coamplification 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 nine 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 were 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 (six of 16) and *EGFR* amplification (four of 16) among the cases with *Myc* amplification were highly statistically significantly (χ^2 : $P=0.00019$ and 0.0017 , respectively).

Coamplification 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 coamplification of *ERBB2* or *EGFR* with *Myc* occurred in gastric cancers but also the colocalization of the coamplified 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 coamplification of non-syntenic 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), whereas those in DMs appeared as 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), whereas DMs are explained by looping out (LO) of extrachromosomal 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 intrachromosomally 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 coamplified cells consisted as a single major population, although the

amplification types and copy numbers of coamplified 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 coamplified 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 that the two genes are very close together, and probably are located on the same amplicons. A possible mechanism of the coamplification 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 Figure 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 non-syntenic 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 coamplified on two HSRs in the breast cancer cell line MDA-MB-134. Similarly, clustered coamplification 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

Speculated mechanism of the coamplification

Figure 4 The proposed mechanisms leading to a coamplification of *Myc* with either *ERBB2* or *EGFR*.

that the two genes come closely together by translocation before starting amplification by BFB or LO, as shown in Figure 4.

The different amplification types ('HSR' type or 'DM' type) and/or the different copy number of coamplified 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 Figure 4. Actually, *ERBB2* and *TOP2A*, which reside very close to each other at chromosome 17q12³⁶ and are separated by approximately 690 kb (NCBI human genome database), are occasionally coamplified in breast and gastric cancers. Similarly, *KIT*, *VEGFR2*, and *PDGFR*, originally located adjacently within a range of approximately 360 kb on chromosome 4q11–13, are coamplified in some glioblastomas.³⁷ However, in either tumors, dual-color FISH images of coamplified 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, coamplification 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 coamplifications. 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, although patients with breast cancer with coamplification 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 proapoptotic function of dysregulated MYC counterbalanced by antiapoptotic signals provided by amplified *ERBB2* is set free by trastuzumab.⁴²

In 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 coamplification of *Myc* with *ERBB2* or *EGFR* occurred non-incidentally, (ii) this coamplification occurred in single cells, and (iii) this coamplification 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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