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Original contribution

Amplicons in breast cancers analyzed by multiplex ligation-dependent probe amplification and fluorescence in situ hybridization☆,☆☆

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Summary Gene amplification is a common event in breast cancer, and identifies actual and potential targets of molecular therapy. The aim of the present study was to determine the amplification status of 22 genes that are reportedly frequently amplified in breast cancers. An archive of 322 formalin-fixed and paraffin-embedded invasive breast cancer tissues were screened by multiple ligation-dependent probe amplification (MLPA) and a total of 906 gene loci judged as 'gain' or 'amplified' was further confirmed to have been amplified based on fluorescence in situ hybridization (FISH). The results showed that 109 of 322 tumors (34%) displayed gene amplification of at least one of the 22 genes. The frequencies of the amplification of four regions containing known driver oncogenes were as follows: 8p11 (*ZNF703*, *FGFR1*, *ADAM9*, *IKBKB*), 8q24 (*MYC*), 11q13 (*CCND1, C11ORF30*), and 17q11–21 (*CPD*, *MED1*, *ERBB2*, *CDC6*, *TOP2A*, *MAPT*) exhibited amplification in 9.6%, 9.6%, 12.4%, and 12.1% of the tumors, respectively. In addition to homogeneously staining region- or double-minute chromosome-type amplifications, a centromere-associated-type amplification was found in nine tumors. Co-localization of the amplicon on 8p11 and the amplicon on 11q13 in single cells was found in 10 tumors, and in six of those tumors the two amplicons constituted single amplification units. Similarly, an amplicon consisting of *ERBB2* and its flanking genes on 17q12–21 co-localized with an amplicon on 8p11 in 10 tumors and with the amplicon on 11q13 in five tumors. Thus, precise and feasible analysis of gene amplification status can be obtained using a combination of MLPA and FISH.

☆ This laboratory study was approved by the Institutional Review Board at the Kanazawa University Hospital [Approval No. 265] and written informed consent was obtained from all patients.

☆☆ Competing interests: No conflicts of interests are declared.

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1. Introduction

Gene amplification is defined as an increase in the copy number of a restricted region of a chromosome arm, and the amplified DNA segment (amplicon) in cancer can attain a size of tens of megabases [1], covering multiple genes [2]. The levels of amplification can range from a single additional copy to more than a hundred copies. Analysis of amplified DNA in mammalian cell lines and tumors has revealed that high-level gene amplification can occur either intrachromosomally (in the form of homogeneously staining regions, HSRs) or extrachromosomally (as double-minute chromosomes, DMs). It is also known that fluorescence in situ hybridization (FISH) of interphase nuclei detects the amplified gene of interest in HSRs as one or more large clusters of signals, whereas those in DMs are detected as multiple scattered signals [3]. Although little is known about the mechanism of amplification or, in particular, about the processes that initiate gene amplification [4], at least two distinct initiating mechanisms have been proposed. Specifically, HSRs appear to be formed by breakage-fusion-bridge (BFB) cycles, whereas DMs are believed to result from "looping out" of extrachromosomal sequences. The BFB cycle consists of a series of recombination events and is physically initiated by a chromosome break. An initial break can occur on the telomeric side; such a break would occur following the fusion of broken chromatid ends, an event that leads to the formation of dicentromeric chromosomes. If the first fusions occur between chromatids of a single chromosome, the subsequent amplification would be entopical amplification (occurring or situated at the original chromosomal site, as opposed to ectopic amplification) [5]; if the first fusions occur between chromatids of different chromosomes, the subsequent amplification would produce chromosomal translocation and could lead to ectopic amplification [4]. Dicentromeric chromosomes then are separated by a second break; thus, intrachromosomal amplification has been speculated to depend on breakage of a given chromosome at a minimum of two sites. At least in the initial stages [6], the size and extent of the amplicon is determined by the distance between the break sites. In contrast, in clinical tumors it has been speculated that amplicon status changes in the process of cancer progression.

Gene amplification is a common event in breast cancers, with 60% of such cancers reportedly showing HSRs [7]. Among the common amplicons are those located at chromosome regions 8p11, 8q24, 11q13, and 17q11–21 [8,9], which contain established driver oncogenes. For instance, *ERBB2* (also known as *HER2*) maps to chromosomal region 17q12; the cyclin D1-encoding gene (*CCND1*) maps to 11q13; *MYC* maps to 8q24.1; and *FGFR1*, which is thought (by some) to be a driver of cancer, maps to 8p11 [10,11].

The aim of the present study was to clarify the extent and copy number of the amplicons commonly found in breast cancers. This work focused on the chromosome regions noted above, given that these amplicons contain known or potential targets of therapy. For this purpose, we screened an archive of formalin-fixed and paraffin-embedded (FFPE) invasive breast cancer specimens using multiplex ligation-dependent probe amplification (MLPA).

2. Materials and methods

2.1. Patients and control cell lines

Four hundred invasive breast cancers obtained from operations at the Department of Surgery in Kanazawa University Hospital between 2010 and 2014 were used. According to the recommendation of the 13th St. Gallen International Breast Cancer Conference (2013) Expert Panel, immunohistochemical (IHC) tests for estrogen receptor (cut-off of 1% of positive nuclei), progesterone receptor (cut-off of 1% of positive nuclei), and Ki-67 (cut-off of 30% of positive nuclei), together with 3 + IHC for ERBB2 (HercepTest™) (Agilent Technology, Santa Clara, CA) or in situ hybridization tests for *ERBB2* amplification were used to classify the tumors into five clinicopathologic surrogate subtypes [12], as follows: Luminal A–like (LA), Luminal B–like/HER2-negative (LB-), Luminal B–like/HER2-positive (LB+), HER2-positive and non-luminal (HER2), and triple negative (TN).

A total of 322 tumors, excluding 78 tumors subjected to preoperative chemotherapies, were examined. Serial sections cut from representative FFPE cancer specimens were used for MLPA and FISH. In the surgeries of 293 patients, the MAS–coated slides™ (Matsunami, Osaka, Japan) were touched to the cancer tissue, dried, and fixed immediately in methacarn solution (methanol/acetic acid, 3:1), dehydrated in a series of ethanol solutions, and stored in a freezer pending FISH analysis.

2.2. MLPA

DNA was subjected to MLPA using the MLPA P078-C1 Breast tumor kit (MRC-Holland, Amsterdam, the Netherlands), which contains one to four probes for each of 22 established cancer-related genes, as shown in Table 1. The resulting PCR products were separated on an ABI-310 capillary sequencer (Applied Biosystems, Foster City, CA) and the results were interpreted using GeneMapper software (Applied Biosystems). Data analysis was performed with Coffalyser MLPA-DAT software version 9.4 (MRC-Holland) to normalize peak values. The test was performed in duplicate, and the arithmetic mean of all the probe peaks was calculated. Average peak values were defined as follows: above 2.0, 'amplified'; between 1.3 and 2.0, 'gain'; between 0.7 and 1.3, 'normal'; below 0.7, 'lost'. Both 'amplified' and 'gain' results were considered MLPA-positive.

2.3. FISH

The FISH protocols for FFPE tissues and imprinted cancer cells were as described previously [13]. All BAC probes were mapped to the UCSC Genome Browser assembly

Table 1 Comparison of MLPA and FISH

Name of gene	Chromosomal locus of gene	FISH probe	MLPA	Clinico-pathological surrogate subtype					Total
				LA	L B-	LB+	HER2	TN	
ESR1	6q25.1	*RP11-450E24*	Amp	0/0	0/0	1/1	0/0	0/0	1/1
			Gain	2/2	3/4	0/0	0/1	0/1	5/8
EGFR	7p11.2	*RP11-339F13*	Amp	0/0	0/0	0/0	1/1	0/0	1/1
			Gain	0/4	0/2	0/0	0/1	0/1	0/8
ZNF703	8p11.23	*RP11-762E21*	Amp	13/19	3/5	5/5	2/2	0/0	23/31
			Gain	1/67	0/14	1/5	0/2	0/9	2/97
FGFR1	8p11.22	*RP11-148D21*	Amp	15/20	2/2	5/5	0/0	0/0	22/27
			Gain	0/9	0/3	2/3	0/0	0/4	2/19
ADAM9	8p11.22	*RP11-60 N22*	Amp	6/9	2/3	0/0	0/0	0/0	8/12
			Gain	7/16	0/3	2/3	0/1	0/0	9/23
IKBKB	8p11.22	*RP11-761H8*	Amp	6/8	0/1	0/0	0/0	0/0	6/9
			Gain	2/16	2/11	0/1	0/1	0/2	4/31
PRDM14	8p13.3	*RP11-152C15*	Amp	2/3	2/2	0/0	0/0	0/0	4/5
			Gain	1/25	0/14	0/4	0/6	0/4	1/53
MTDH	8q22.1	*RP11-662P7*	Amp	3/4	2/2	1/1	0/0	1/2	7/9
			Gain	5/31	0/18	3/3	0/0	0/4	8/56
MYC	8q24.21	*RP11-440 N18*	Amp	5/6	5/6	3/3	0/0	3/3	16/18
			Gain	6/41	2/14	1/9	2/6	2/10	13/80
CCND1	11q13.3	*RP11-300I6*	Amp	17/19	10/10	4/4	1/1	0/0	32/34
			Gain	3/26	1/9	2/5	1/3	0/4	7/47
C11ORF30	11q13.5	*CTD-2501F13*	Amp	7/8	3/3	2/2	0/0	0/0	12/13
			Gain	2/5	2/4	2/2	1/1	0/0	7/12
CDH1	16q22.1	*RP11-354 N7*	Amp	0/0	0/0	0/0	1/1	0/0	1/1
			Gain	0/1	0/2	0/0	0/0	0/3	0/6
			Lost	2/11	2/3	2/2	0/0	0/1	6/17
CPD	17q11.2	*RP11-146A4*	Amp	0/0	0/0	2/2	3/3	0/0	5/5
			Gain	0/2	0/2	3/3	1/1	0/1	4/9
MED1	17q12	*RP11-916F3*	Amp	0/0	0/0	8/8	10/10	0/0	18/18
			Gain	0/11	0/6	6/8	1/2	0/2	7/29
ERBB2	17q12	*RP11-62 N23*	Amp	0/0	0/0	13/13	18/18	0/0	31/31
			Gain	0/15	0/5	5/5	3/3	0/1	8/29
CDC6	17q21.2	*RP11-175 M14*	Amp	0/0	0/0	0/0	6/6	0/0	6/6
			Gain	0/7	1/5	5/8	2/4	0/1	8/25
TOP2A	17q21.2	*RP11-259G21*	Amp	0/0	0/0	0/0	3/3	0/0	3/3
			Gain	0/7	1/5	4/6	4/7	0/1	9/26
MAPT	17q21.31	*RP11-769P22*	Amp	0/0	0/0	0/0	1/1	0/0	1/1
			Gain	0/4	0/4	0/0	0/0	0/0	0/8
PPM1D	17q23.2	*RP11-67D12*	Amp	1/1	2/2	4/4	3/3	0/0	10/10
			Gain	0/13	0/6	0/1	0/3	0/5	0/28
BIRC5	17q25	*RP11-116B21*	Amp	1/3	0/0	2/2	0/0	0/0	3/5
			Gain	0/17	1/8	1/3	1/4	0/4	3/36
CCNE1	19q12	*RP11-345 J21*	Amp	0/0	0/0	0/0	0/0	1/1	1/1
			Gain	0/1	1/3	0/1	1/1	0/4	2/10
AURKA	20q13.31	*RP5-1167H4*	Amp	0/0	0/0	0/0	0/0	0/0	0/0
			Gain	2/15	0/5	0/2	0/1	0/2	2/25
No. of examined cases				209	47	22	19	25	322
No. of Amp-positive cases				43	21	22	19	4	109

NOTE. Values are presented as the number of cases with gene amplification validated by FISH (numerators) divided by the numbers of cases with 'amplified' or 'gain' cases classified by MLPA (denominators).
Abbreviations: LA, Luminal A–like; LB-, Luminal B–like/HER2-negative; LB+, Luminal B–like/HER2-positive; HER2, HER2-positive; TN, triple negative; Amp,'amplified' by MLPA; Gain, 'gain' by MLPA.

ID: hg38 or 19 (UCSC Genome Browser. http://genome.ucsc.edu/). All FISH probes used in this study are summarized in Table 1. All but one was acquired from BACPAC Resources (Oakland, CA); the sole exception was CTD-2501F13, which was obtained from Thermo Fisher Scientific (Waltham, MA). The probes were labeled with SpectrumOrange™ or

SpectrumGreen™ using a nick translation kit (Abbott Laboratories, Abbott Park, IL). For quantification of gene amplification, DNA probes specific for the alpha satellite DNA of the centromeric regions of each chromosome 6, 7, 8, 11, 16, 17, and 20 (CEP™ 6, 7, 8, 11, 16, 17, and 20) [14] were purchased from Abbott Laboratories and were co-hybridized to standardize the chromosome number. As a DNA probe specific to the centromere region of chromosome 19 is not commercially available, a BAC probe specific for the peri-centromeric region of chromosome 19 (RP11-587H3) was used instead.

Tumors demonstrating co-amplification of multiple genes were further examined by simultaneous hybridization using two separate probes (labeled with distinct fluorescent markers) against the genes of interest, permitting assessment of the co-existence of the amplified genes or chromosomal regions in single nuclei or single amplicons. Removal of protein from the tissue sections, denaturation, hybridization, and post-hybridization washing were performed as described previously [13]. 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™.

Scoring and evaluation of FISH slides was performed manually by counting the target gene signals and control signals in 40 tumor cell nuclei per sample. Gene amplification was determined by modifying the updated guideline for HER2 FISH categories of the American Society of Clinical Oncology/College of American Pathologists criteria [15], as follows. Classical amplification status was assigned to specimens that exhibited both a ratio of gene/chromosome ≥2.0 and a ratio of mean gene/cell ≥6.0. These specimens were further classified as HSR type if the amplified signals were clustered, as DM type if the amplified gene signals were scattered, or as mixed (MX) type if both types of signal were observed. 'Co-amplified/polysomy' (CoPoly) was assigned to specimens that exhibited a gene/chromosome ratio< 2.0 and a mean gene/cell ratio ≥ 6; 'monosomy' (Mono) was assigned to specimens that exhibited respective ratios of ≥2.0 and< 4.0. Specimens that yielded one or more additional copies of genes compared to control signals and were not sorted into the above categories were originally defined as low-level amplification (LA).

3. Results

3.1. MLPA

MLPA analyses were performed on 322 of the FFPE breast cancer samples. The 7084 gene loci of the 322 tumors were categorized as 241 'amplified', 665 'gain', 6161 'normal', and 17 'lost' genes, as shown in Table 1. Although there was an overall good agreement between MLPA and FISH data, the concordance rate varied among the respective genes, as shown in Table 1. Concerning *ERBB2*, there were two cases in which the *ERBB2* MLPA results were discordant with those obtained by IHC and FISH: specifically, one 'LB+' tumor and one 'HER2' tumor showed 'normal' *ERBB2* values by MLPA. In total, 109 of 322 tumors (33.9%) displayed FISH-confirmed gene amplification of at least one of the 22 examined genes.

3.2. FISH

Although no significant differences were found between the results of FISH performed on FFPE sections and on imprinted cells, analysis with higher resolution and more precise enumeration was possible in the latter compared to the former, as shown in Fig. 1 (compare A and B, C and D, and G and H, respectively).

3.3. 8p

Amplification of the genes located on 8p and 8q is summarized in Fig. 2A. Amplifications of *FGFR1* and *ZNF703* were observed at similar frequencies. By dual-color FISH, the signals for *ZNF703, FGFR1, ADAM5*, and *IKBKB*, if amplified, were found to be closely associated, suggesting location on the same amplicons. The observed amplicons were of various types, including HSR (Fig. 1A), CoPoly (Fig. 1C), LA (Fig. 1G), and Mono (Fig. 1I). However, except for eight cases of HSR type, all of the amplicons exhibited the PC subtype (Fig. 2A), and thus were considered to be entopically located. In Case 8 (Fig. 1C-F), the tumor nuclei had large signals for centromere 8 surrounded by numerous amplified gene signals for 8p (Fig. 1D, large arrows); this specimen also yielded small paired signals for the 8p genes and centromere 8 (Fig. 1D, small arrows).

3.4. 8q

Although *MYC* also was amplified in a variety of amplicon types, DM (Fig. 1J) and CoPoly types were observed the most frequently (Fig. 2A). The PC subtype was detected only in two LA-type and one CoPoly amplicons. In Case 53, tight or loosely clustered signals for *MYC* and centromere 8 were observed (Fig. 1K and L). Co-localization of *MYC* and *MTDH*, and of *MYC* and *PRDM14*, in single nuclei were found in ten and four cases, respectively. Co-amplification of the genes located on 8p and 8q was observed in seven tumors; however, in each case the two amplicons were separated.

3.5. 11q13

The amplification of *CCND1* was found in 40 tumors; 31 of these tumors exhibited the HSR type and 21 of the HSR-type amplicons were of the PC subtype (Fig. 2B). In Cases 69 and 76, increased numbers of *CCND1* signals associated with small signals for centromere 11 were observed, as shown

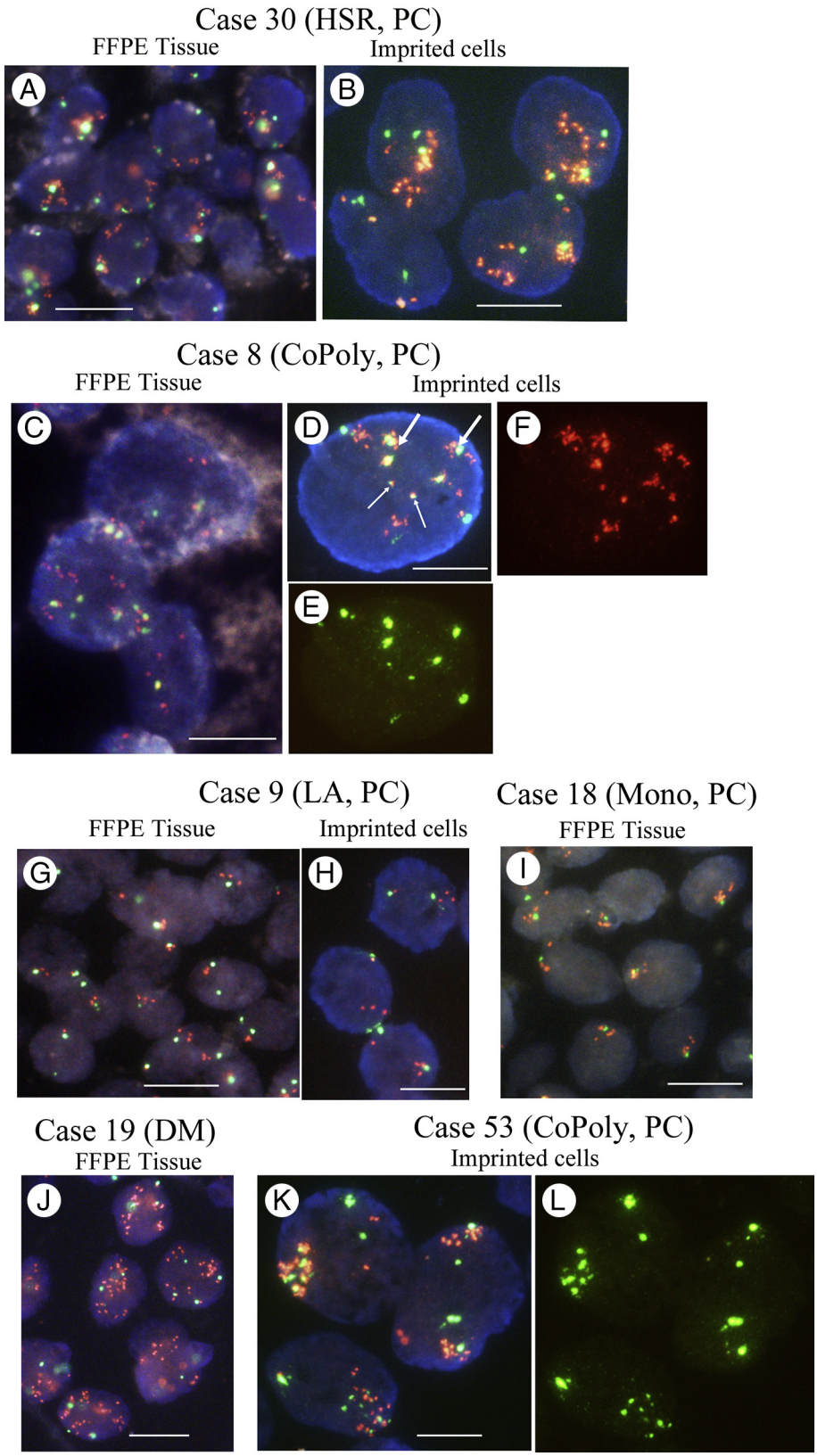

Fig. 1 Amplifications of *FGFR1* (Cases 30, 8, and 9) (A-H), *ZNF703* (Case 18) (I), and *MYC* (Cases 19 and 53) (J-L) (orange, gene-specific signals; green, signals for the centromeric regions on which the specific genes are located). In Panel D, paired signals of *FGFR1* and centromere 8 (small arrows) and large signals of centromere 8 surrounded by numerous *FGFR1* (large arrows) signals were observed (D, triple-band filter; E, SpectrumGreen™-specific filter; F, SpectrumOrange™-specific filter). Panels K and L showed co-amplification of *MYC* and centromere 8. PC: PC-subtype. Scale bar: 10 μm.

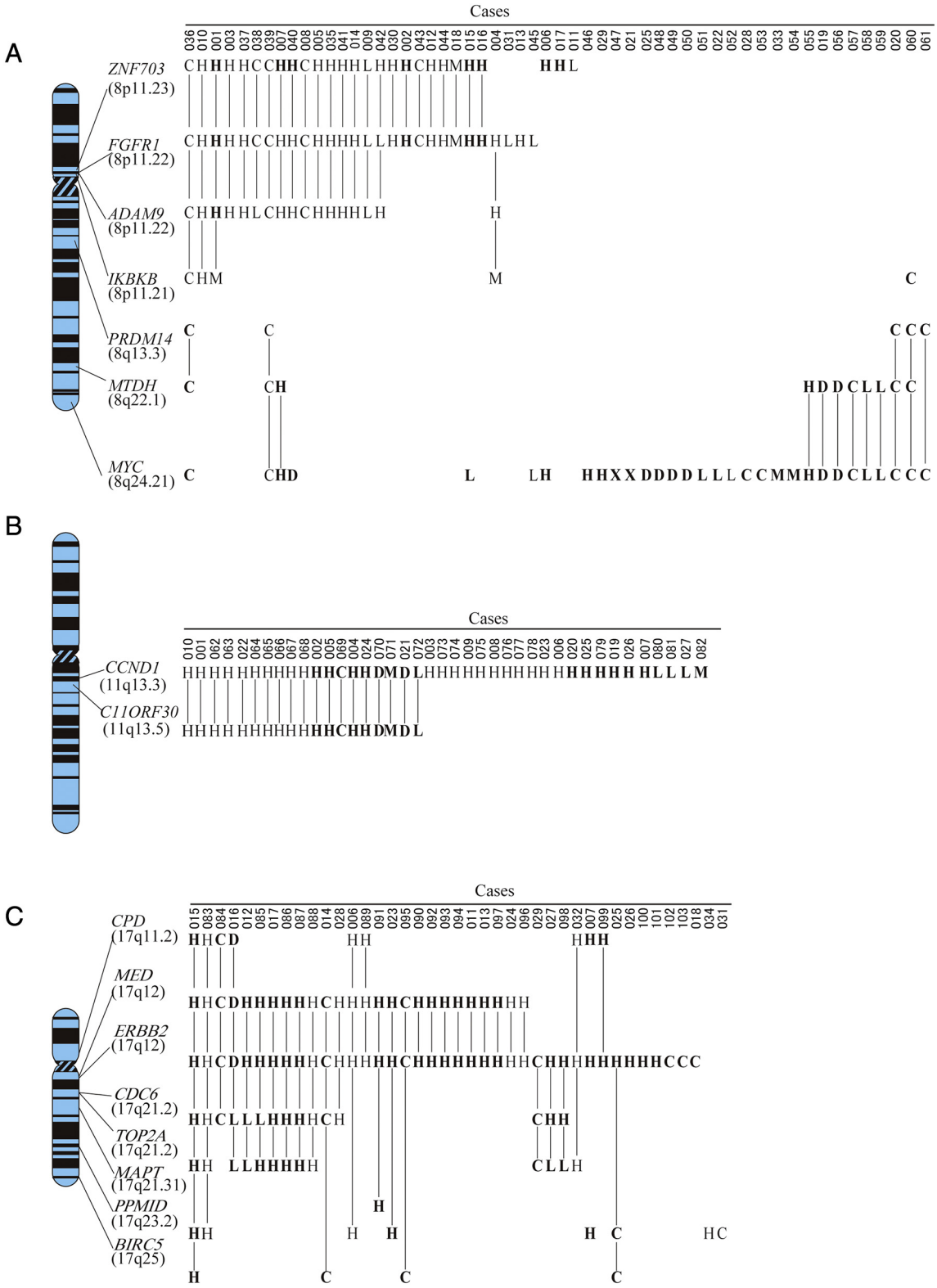

Fig. 2 Amplified genes in chromosomes 8p and 8q (A), 11q (B), and 17q (C). Abbreviations: H, HSR type; C, CoPoly type; D, DM type; M, Mono type; L, LA type; X, mixed type. Bold, non-PC type; normal, PC type. Characters of co-amplified genes are connected by solid lines. Scheme of Giemsa banding of the chromosomes is based on ISCN (1991) [24].

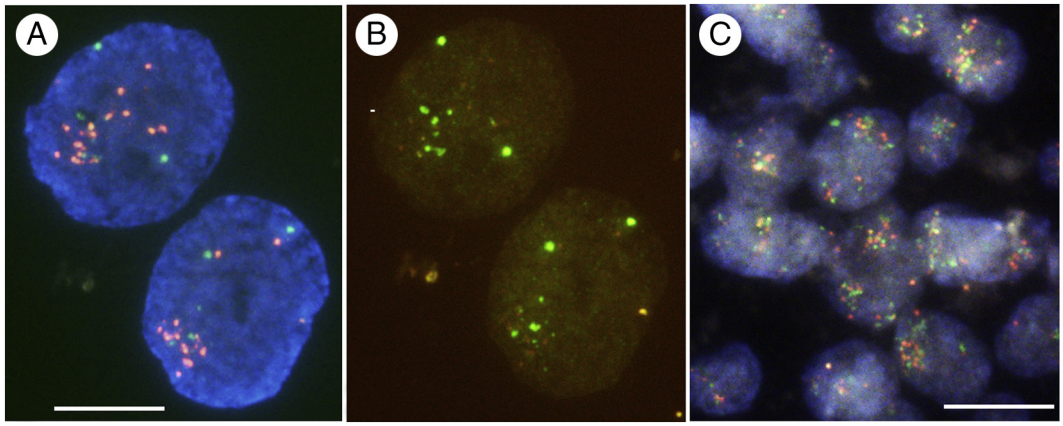

Fig. 3 Amplicon on 11q13. Overlapped loose clusters of *CCND1* (orange) and small signals of centromere 11 (green) were found in the imprinted cells (A, triple-band filter; B, SpectrumGreen™-specific filter; Case 69). Dual-color FISH on FFPE tissue shows co-localization of *CCND1* (green signals) and *C11ORF30* (orange signals) (C, Case 4). Scale bar: 10 μm.

in Fig. 3A and B. Among tumors that yielded amplification of *CCND1*, 19 of 40 (48%) exhibited co-amplification of *C11ORF30* (Fig. 3C). Conversely, no amplification of *C11ORF30* was observed without co-amplification of *CCND1*.

3.6. 17q (17q12–21)

The HSR-type amplification of *ERBB2* was found in 31 tumors (Fig. 2C). In eight of these 31, the clustered *ERBB2* signals were found near CEP17 signals (PC subtype); in the other

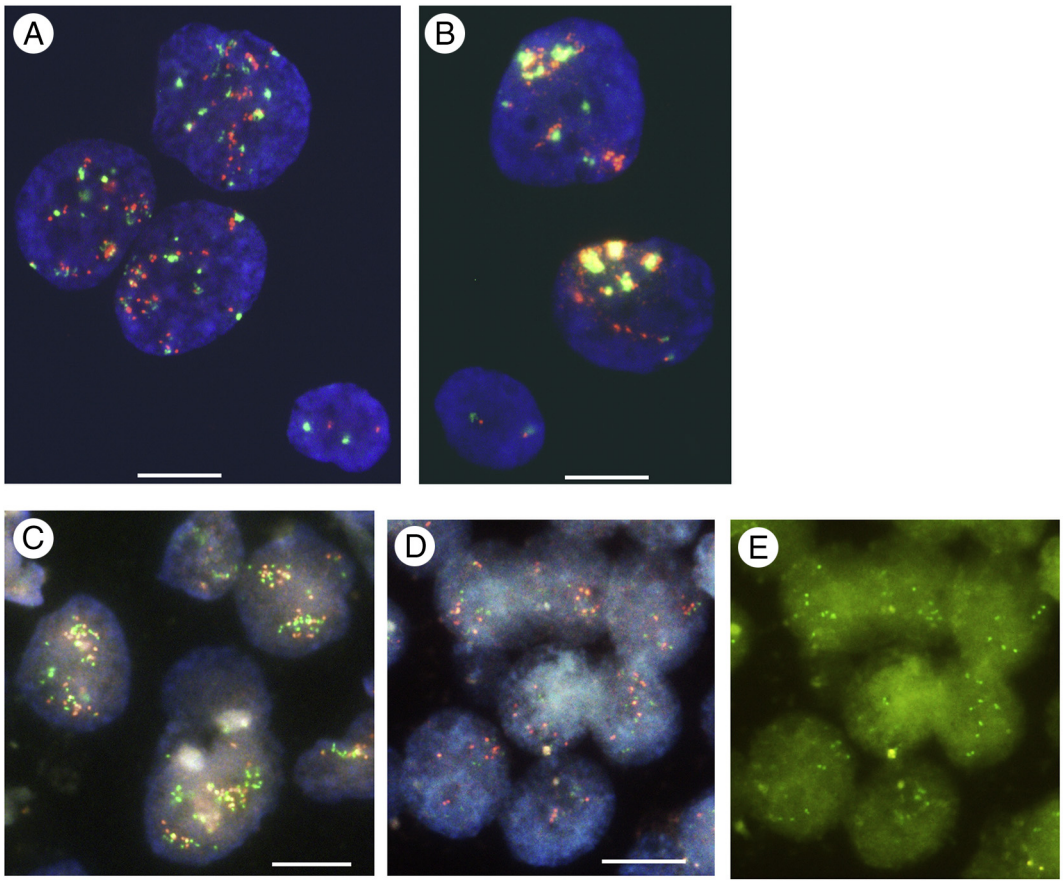

Fig. 4 Amplicon on 17q. CoPoly-type, PC-subtype amplification of *ERBB2* (orange) and centromere 17 (green) on imprinted cells (A, Case 103; B, Case 29). Numerous minute paired signals were found in (A); small *ERBB2* signals aggregated around the large centromeric signals were found in (B). Normal lymphocytes with two paired signals are seen at the corners. Note the co-amplification of *CPD* (orange) and *ERBB2* (green) in Case 83 (C), and of *BIR5* (orange) and *ERBB2* (green) in Case 14 (D, triple-band filter: E, SpectrumGreen™-specific filter). Scale bar: 10 μm.

Table 2 Co-amplification of non-syntenic genes

		Case No.																																
Name of gene	Chromosomal locus of gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
ESR1	6q25.1																														H	L			L	
ZNF703	8p11.23	**H**	**H**	H		H	**H**	**H**	H	L	H	L	H		H	**H**	**H**	**H**	M												H					H
FGFR1	8p11.23	**H**	**H**	H	H	H		**H**	H	L	H		H	H	H	**H**	**H**		M												H	L				H
ADAM9	8p11.22	**H**		H	H	H		**H**	H	L	H				H																					H
IKBKB	8p11.21	**L**	**H**		M						H																									H
PRDM14	8q13.3																				**C**															
MTDH	8q22.1							**H**												**D**	**C**															
MYC	8q24.21						**H**	**H**												**D**	**C**	**X**	**L**						**C**	**H**				**M**		
CCND1	11q13.3	H	**H**	H	**H**	**H**	H	**H**	H	H	H									**H**	**H**	**D**	H	H	**H**	**H**	**H**	**L**								
C11ORF30	11q13.5	H	**H**		**H**	**H**					H											**D**	H		**H**											
CDH1	16q22.1																									**H**										
CPD	17q12						H	**H**								**H**	**D**																			
MED1	17q12						H					**H**	**H**	**H**	**C**	**H**	**D**	**H**						**H**	H				H							
ERBB2	17q12						H	**H**				**H**	**H**	**H**	**C**	**H**	**D**	**H**	**C**					**H**	H	**H**	**H**	**H**	H	**C**			H			
CDC6	17q21.2												**L**		**C**	**H**	**L**	**H**										**H**	H	**C**						
TOP2A	17q21.2												**L**			**H**	**L**	**H**										**L**		**C**			H			
PPM1D	17q23.2						H	**H**								**H**								**H**		**C**						C			H	
BIRC5	17q25														**C**	**H**										**C**										
CCNE1	19q12																																H	**C**		
AURKA	20q13.31																																			**L**

Note. Bold, amplification in non-PC subtype; normal, amplification in PC subtype.

Abbreviations: H, HSR-type amplification; L, Low-level amplification; M, Monosomy-type amplification; D, DM-type amplification; C, Co-amplified/polysomy-type amplification; X, Mixed HSR- and DM-type amplification.

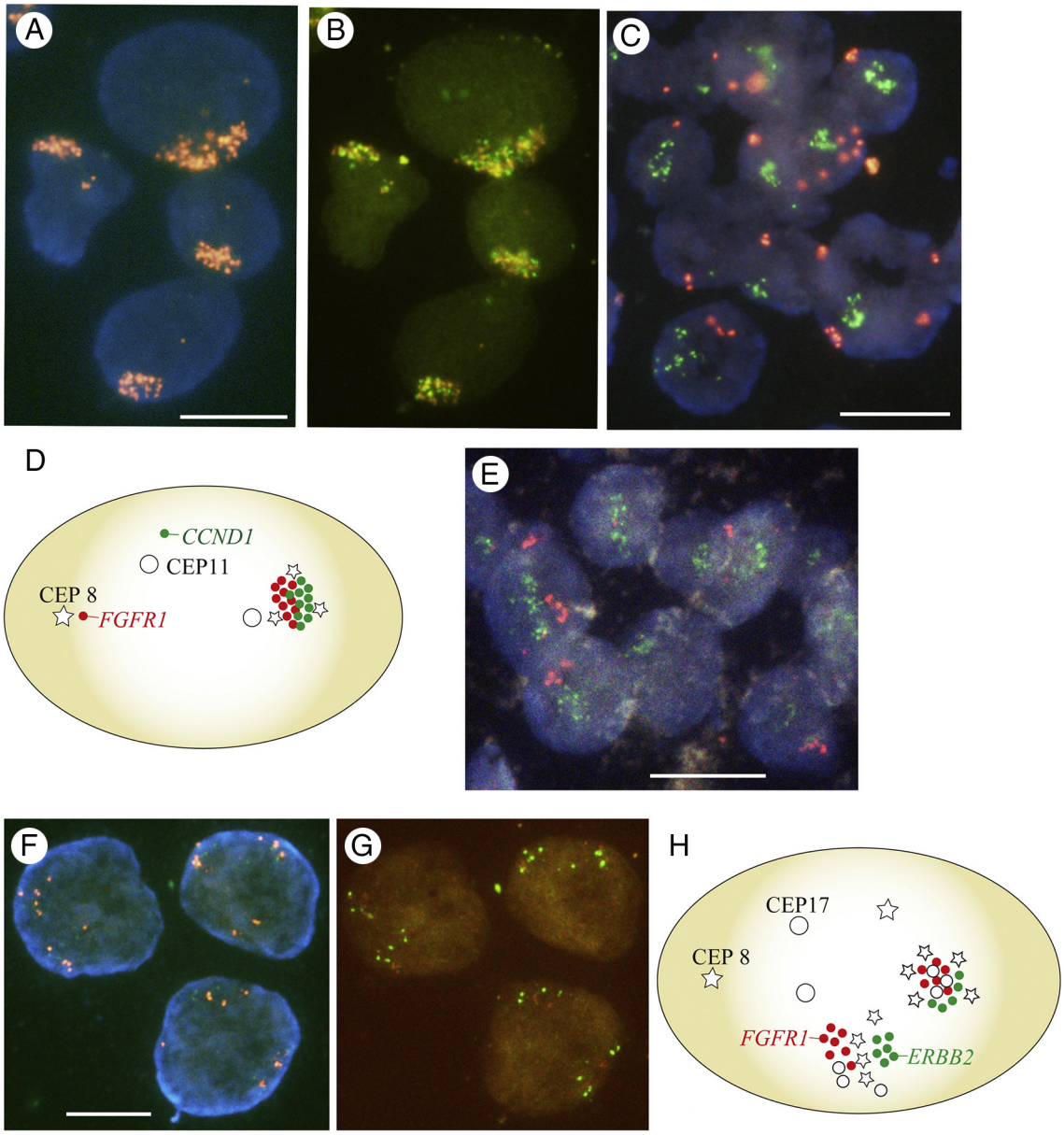

Fig. 5 Amplicons containing non-syntenic genes. Dual-color FISH on imprinted cells shows superimposed signals for *FGFR1* (orange) and *CCND1* (green) in Case 3 (A, triple-band filter; B, SpectrumGreen™-specific filter). In Case 7 (C), co-amplification of *ZNF703* (orange) and *CCND1* (green) was observed in single nuclei but in different amplicons. Panel D shows a schematic diagram of the amplification status of Case 3. Clustered signals for *FGFR1* (orange) and *ERBB2* (green) were found separated in Case 13 (E), but in close association in Case 14 (F, triple-band filter; G, SpectrumGreen™-specific filter). Panel H shows a schematic diagram of the amplification status of Case 14. Scale bar: 10 μm.

23 tumors, the clustered *ERBB2* signals were not localized with the CEP17 signals, suggesting that these amplicons were located ectopically. The CoPoly type was found in seven tumors where mean (per cell) signals for both *ERBB2* and CEP17 exceeded 10. In two of these seven specimens, the amplified signals of *ERBB2* and CEP17 were separated (Cases 18 and 89). In the other five of these seven specimens, paired signals for *ERBB2* and CEP17 were found scattered (Fig. 4A) or clustered (Fig. 4B), in different nuclei or in single nuclei. In Case 29, a strong centromere-17 signal was surrounded by numerous *ERBB2* signals, as shown in Fig. 4B.

The amplified genes flanking *ERBB2*, such as *MED1, CDC6*, and *TOP2A*, were frequently co-localized with amplified *ERBB2*; the frequency of amplification gradually decreased with distance from *ERBB2* (Fig. 2C). *ERBB2* and the genes more remote from *ERBB2* on 17p (such as *CPD, PPM1D*, and *BIRC5*) were occasionally co-amplified in the same amplicon, as shown in Fig. 4C-E. Case 7 presented a

unique example, wherein *CPD* and *PPM1D* were co-amplified with *ERBB2* in single nuclei but did not co-amplify with each other.

3.7. Others

The amplification of *ESR1* (6q25) was observed in six tumors including a case with an incidental metaphase spread, where four copies of *ESR1* were seen arranged in tandem near a centromere-6 signal (Supplementary Fig. 1). *CCNE1* (19q12) was amplified in three cases: one such case exhibited the HSR type and the other two exhibited the LA type. Amplification of *AURKA* (20q13.2) was found in two cases: one such case exhibited the CoPoly type and the other exhibited the LA type. *EGFR* (7p11.2) and *CDH1* (16q22.1) were amplified in HSR in a separate case.

3.8. Co-amplifications of non-syntenic genes (genes physically located on different chromosomes)

Co-amplification of non-syntenic genes in single nuclei was found in 35 tumors, as listed in Table 2. Dual-color FISH revealed co-localization of an amplicon on 8p11 (consisting of *ZNF703, FGFR1, ADAM9*, and/or *IKBKB*) and an amplicon on 11q13 (including *CCND1* with or without *C11ORF30*) in single cells in 10 tumors. In six of these specimens (Cases 1–6), the two amplicons were thought to constitute a single amplification unit because signals for the genes representing both amplicons were superimposed, as shown in Fig. 5A and B. However, in the other four of these 10 tumors, the signals for the two amplicons were separately distributed in individual nuclei, as shown in Fig. 5C. In Cases 3 and 4, signals for centromeres 8 and 11 were detected in the amplification unit (Supplementary Fig. 2). A possible amplification status for Case 3, as inferred from these dual-color FISH findings on touch smears, is depicted schematically in Fig. 5D. Unfortunately, touch smears were not obtained for Case 4, precluding this sort of precise analysis.

The amplicon containing *ERBB2* and its flanking genes was co-localized with the amplicon on 8p11 in eight tumors, and with the amplicon on 11q13 in seven tumors, as shown in Table 2. In these tumors (excluding Case 14), the two amplicons were observed separately in individual nuclei (Fig. 5E). In Case 14, dual-color FISH on the touch smear revealed not only representative signals for the two amplicons (as shown in Fig. 5F and G) but also clustered signals for centromeres 8 and 11 (as shown in Supplementary Fig. 3). Based on these findings, the amplification status of Case 14 is inferred to be as depicted schematically in Fig. 5H.

In Case 25, clustered signals for the amplicon on 17q were co-amplified with *CDH1*, a gene originally located at 16q22.1 (Supplementary Fig. 4). Other co-amplifications of non-syntenic genes were observed in single nuclei but appeared to correspond to different amplicons.

4. Discussion

In the present study, each of the 22 genes examined were found to be amplified in at least one of the tumor samples, although the frequency of detection of each gene varied. Some of these genes are established driver oncogenes that have already been tested as clinical targets of molecular therapies; others are candidate oncogenes that are being targeted by therapies that are the subject of ongoing clinical trials or are in development. The targeted therapy against amplified *ERBB2* has been the most successful molecular therapy in treating breast cancer. Clinical studies using FGFR1-selective inhibitors also have been performed [10,16-18]. CCND1 is generally regarded as difficult to target directly by therapies; however, CCND1's partner kinases, such as cyclin-dependent kinases 4 and 6, are considered better targets [10,19,20]. Therapies targeting *IKBKB* and *ADAM9* by the use of specific micro-RNAs are under investigation and remain the subject of *in vitro* studies [21,22]. ZNF703, the product of one of the genes most frequently amplified on 18p11, also is considered a promising candidate for molecular targeting [11]. In addition, although TOP2A is not a molecular target, *TOP2A* amplification is a biomarker that predicts chemo-sensitivity to anthracyclines [2]. Multiple other genes on the various amplicons also may have significant roles in breast cancer and so represent potential targets for therapy.

Recently, a Phase II study evaluating the efficacy of dovitinib, a potent FGFR inhibitor, against breast cancer terminated early because of slow accrual of patients with amplifications of *FGFR1*, *FGFR2*, and *FGFR3* [17]. To avoid delays in the recruitment of patients with rare molecular markers, molecular screening using next-generation sequencing assays [17] or droplet digital PCR analyses using circulating tumor DNA [16] have been proposed. However, compared to these methods, MLPA is a relatively cheap and easy-to-perform PCR assay that allows simultaneous detection of multiple gene copy-number aberrations from small amounts of fragmented DNA derived from formalin-fixed material.

We also observed the close association of centromere signals with signals for the amplicons; specifically, the amplicons on 17q12–21, 11q13, 8p11, and 8q24 were closely associated with the respective centromere signals in five, two, one, and one tumors, respectively (Figs. 1D-F, K and L, and 4A and B). One such image appeared to show co-amplification of the *ERBB2* sequence with centromere 17; this image is of particular interest because balanced increases in the numbers of *ERBB2* and centromere 17 would have been interpreted as polysomy 17 by the 2007 criteria and so would have been excluded as a potential target of trastuzumab. However, more recent criteria suggest that increases in centromere-17 copy number may not necessarily represent chromosome-17 polysomy but instead may correspond to gain or amplification of the chromosome-17 centromeric region [23]. The present study suggested that gene amplification associated with centromeric regions may be a common type of amplification event, occurring as a subset of nearly all common amplifications.

Based the previous studies delineating the boundaries of the amplicons, common initial break sites in the amplicons are known to correspond to specific genes. Initially, genes located near each other in a chromosome region are co-amplified physically, but the amplicons of advanced cancers selected during tumor development may not be the same as the original amplicons, as shown in Fig. 2. In the present study, the predominant type of amplicon found at 8p, 11q, and 17q was HSR, although 83% (26/31) of *MYC* amplifications occurred in non-HSR types. Marotta et al found neither duplicated segments nor fragile sites within the 6-Mb region surrounding the *MYC* oncogene [4], suggesting a different mechanism for *MYC* amplification.

In the current study, co-amplifications of the non-syntenic genes were observed in 35 tumors, as shown in Table 2. In each of eight of these tumors, the non-syntenic genes constituted a single amplification unit (and thus a larger amplicon). Specifically, these events included the amplicons on 8p11 and 11q13 in six tumors; the amplicons on 8p11 and 17q11–12 in one case; and co-amplification of 17q11–12 and 16q22.1 in another case. The FISH images of intermingling of these non-syntenic genes in single clusters suggests to us that these events correspond to the early fusion of both genes by translocation, with subsequent amplification by BFB cycle.

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Supplementary data

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