의학박사 학위논문

Assessment of HER2 status in invasive breast cancer with increased centromere 17 copy number by fluorescence in situ hybridization

형광제자리보합법 상 17 번 염색체 동원체의 수적 증가를 보이는 침윤성 유방암에서 HER2 의 평가

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ABSTRACT

Background: A subset of breast cancers shows increased copy numbers of chromosome 17 centromere on in situ hybridization (ISH). However, recent studies have revealed that true polysomy 17 is a rare event in breast cancer, and that an increased copy number of centromere 17 represents amplification or copy number gain in and around the centromeric region. In such instances, the use of chromosome enumeration probe targeting centromere 17 (CEP17) in HER2 ISH is limited; thus, alternative methods for precise assessment of HER2 status are necessary. Performing ISH using probes for other genes on chromosome 17 as additional reference genes has been proposed by 2013 ASCO/CAP guidelines as well as several previous studies. In this study, we applied this method to breast cancers with increased CEP17 copy numbers (≥ 2.6) and compared it with conventional methods based on the 2007 and 2013 ASCO/CAP guidelines.

Methods: After reviewing all HER2 fluorescence in situ hybridization (FISH) reports recorded from June 2004 to December 2011 at Seoul National University Bundang Hospital, we identified 300 cases (29.6%) with CEP17 copy number ≥ 2.6 from 1013 breast cancers. We performed FISH with

probes for RARA, SMS, and TP53 genes on 253 breast cancers that had available tissue blocks, using tissue microarrays. If one or more gene had a mean copy number <2.6 the largest number for that gene(s) was chosen as an alternative to the CEP17 copy number, and we re-graded the HER2 status based on HER2: alternative gene ratio. After re-grading the HER2 status, we selected 8 cases which represent the various patterns of copy number alterations on chromosome 17, and performed high-resolution array-based comparative genomic hybridization (aCGH) to confirm the genomic copy number variation.

Results: Of the 243 cases in which re-grading were possible, only 2 had copy numbers ≥2.6 for RARA, SMS and TP53 gene. Of 151 breast cancers classified as HER2 non-amplified by the 2007 ASCO/CAP guidelines using the HER2:CEP17 ratio (<1.8), 42 (27.8%) were re-graded as amplified and 33 (21.8%) as equivocal after FISH using additional reference genes. Of the 101 HER2 non-amplified cases by the 2013 ASCO/CAP guidelines, 2 (2.0%) were reclassified as amplified and 24 (23.8%) as equivocal. Of 46 equivocal cases, 35 (76.1%) were re-graded as amplified. After re-grading, amplified cases were significantly increased, and the concordance between HER2 FISH and immunohistochemistry decreased. Of the 8 cases analyzed by aCGH, six were upgraded from non-amplified to amplified by additional FISH studies.

However, only 3 cases were proven to have HER2 amplification on aCGH. Two cases which were assumed to have true polysomy 17 by additional FISH studies were proven not to be polysomic. We also reviewed the pathologic features of the cases whose HER2 status were upgraded to be amplified by additional FISH, but some pathologic features were not matched with those of HER2-amplied tumors.

Conclusion: Using additional reference genes in combination might be an option for accurate HER2 evaluation in breast cancer with increased CEP17 copy numbers. However, it has some limitations. It can cause over-grading of HER2 status, when the tumor has loss of new reference genes. Especially three genes that we used in current study (SMS, TP53 and RARA) were not suitable for alternative reference gene when used independently. Moreover, copy number alterations detected by additional FISH and those by aCGH were not well-correlated. Thus, use of alternative genes on chromosome 17 such as SMS, RARA and TP53 instead of CEP17 is not still suitable to be applied in daily practice. Additional studies to search the most stable gene that rarely shows copy number alteration will be needed.

Keywords: Breast cancer, HER2, Centromere 17, polysomy 17, FISH, reference gene

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LIST OF ABBREVIATIONS

IHC: immunohistochemistry

ISH: in situ hybridization

ASCO/CAP: American Society of Clinical Oncology/College of American Pathologists

FISH: Fluorescence in situ hybridization

CEP17: chromosome enumeration probe 17

IBC: invasive breast cancer

TMA: tissue microarray

FFPE: formalin- fixed, paraffin- embedded

aCGH: array based comparative genomic hybridization

ADM2: Aberration Detection Method 2

TCGA: The Cancer Genome Atlas

CNG: copy number gain

CNL: copy number loss

IDC: invasive ductal carcinoma

NOS: not otherwise specified

ILC: invasive lobular carcinoma

TNBC: triple negative breast cancer

MPLC: metaplastic carcinoma

MPC: micropapillary carcinoma

INTRODUCTION

HER2 is a proto-oncogene that encodes epidermal growth factor receptor with tyrosine kinase activity, located on chromosome 17 at q21 (1, 2). In breast cancers, HER2 protein overexpression is mostly caused by gene amplification, and HER2 amplification is recognized in 15% ~ 20% of invasive breast cancers (IBCs) (3, 4). HER2 amplification is associated with poor prognosis and is a predictive biomarker for response to anthracycline-based chemotherapies (3, 5-8). Most importantly, it is a sole predictive marker for treatment benefits from HER2 targeting agents such as trastuzumab, a humanized monoclonal antibody of HER2 (9). HER2 targeted therapy is exclusively effective for HER2-amplified primary or metastatic breast cancers and thus, a standard of treatment as a single agent or in combination with other chemotherapeutic agents in such cancers (10-14). Therefore, precise assessment of HER2 status is an essential step for treatment of breast cancer.

Immunohistochemistry (IHC) and in situ hybridization (ISH) of HER2 are standard methods for assessing HER2 status (9, 15). American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) recommended performing IHC at the screening step and to carry out ISH to

confirm HER2 amplification if HER2 IHC is equivocal (2+) (9). Fluorescence in situ hybridization (FISH) is the most commonly used in situ hybridization technique. There are 3 HER2 FISH kits approved by FDA: PathVysion (Abbott Molecular, Downers Grove, IL, USA), PharmDx (DAKO, Glostrup, Denmark), and INFORM (Ventana Medical Systems, Tucson, AZ, USA). Among them, PathVysion and PharmDx kits use dual probes-, HER2 and chromosome enumeration probe targeting centromere 17 (CEP17). In the FISH scoring process, correction of specific gene copy numbers with the copy numbers of centromere enumeration probe as a surrogate of chromosome that the gene is located has long been considered crucial (16). By this method, we can compensate for the loss of signals by tissue sectioning and adjust for the natural increase in the number of chromosome during replication. It also helps to detect the chromosomal aneuploidy of tumors (17). Therefore, dual-colored FISH using a CEP is preferred over single-colored.

Chromosome 17 is one of the smallest and most-condensed human chromosomes, and complex structural and numerical aberrations of chromosome 17 have been identified in many genomic based studies (18-20). Aneuploidy of chromosome 17 is frequently observed in breast cancer. We can easily encounter alteration of CEP17 copy numbers during ISH for diagnostic purposes. This phenomenon was thought to result from increasing

number of whole chromosome 17 which is usually referred to as polysomy 17. Until now, there have been no standardized criteria defining polysomy 17. Thus, the reported incidence varied upon the individual criteria used in the studies. ASCO/CAP guidelines in 2007 stated that if polysomy 17 were to be defined as more than 3.0 CEP17 copy numbers, its incidence was approximately 8% of all breast cancers (9).

During recent decades, many researchers have investigated the effect of polysomy 17 on HER2 overexpression as well as response to trastuzumab. Even though there still exist some controversies on this issue, polysomy 17 itself does not seem to be associated with protein overexpression or increased gene dosage of HER2 (21-23) nor does it make any differences in response to trastuzumab (24, 25). However, it is obvious that copy number changes of CEP17 can influence the interpretation of HER2 status defined by ISH using HER2:CEP17 ratio (26-30).

Recent studies have revealed that true polysomy 17 is a very rare event in breast cancers. Yeh et al. reported that there was no true polysomy 17 among 99 cases of breast cancers by comparative genomic hybridization using frozen tissues (31). Moelans et al. also found that true polysomy 17 is a rare event by multiplex ligation-dependent probe study (32). They identified that increased copy number of CEP17 represents amplification or copy number

gain in the centromeric or pericentromeric regions (19, 31-33). It brings into question whether CEP17 is a reliable surrogate for chromosome 17. If the copy numbers of HER and CEP17 are increased together, the HER2:CEP17 ratio can become less than 2 even though HER2 gene copy number itself can be considered amplified. Thus, alternative methods for precise assessment of HER2 status are needed to avoid this delicate problem

ISH using probes of other genes on chromosome 17 as additional reference genes have been proposed by several studies. Toxwell et al. and Marchio et al. proposed the use of SMS and RARA as surrogates for chromosome 17 in breast cancers with altered CEP17, and applied this method to limited number of cases (33, 34). Varga et al. also used RARA, TOP2 and TP53 as surrogates (35). Recently, Tse et al. applied a new method using SMS, RARA, and TP53 on 171 breast cancers with polysomy 17 as additional surrogates and suggested a new HER2 test algorithm (17). In this study, we applied this method to IBCs with increased CEP17 copy numbers (≥ 2.6) and compared it with the conventional method based on 2007 and 2013 ASCO/CAP guidelines.

MATERIALS AND METHODS

1. Patient population and tissue collection

We reviewed all HER2 FISH reports, which had been recorded from June 2004 to December 2011 at Seoul National University Bundang Hospital, to search for IBCs with increased CEP17 copy numbers. In total, 1435 HER2 FISH analyses had been performed. Among them, 1,230 cases were primary or metastatic invasive breast cancers. We excluded 87 cases from outside hospitals and 130 cases in which tissue were obtained by needle biopsy, mammotome excision, or fine needle aspiration. As a result, 1,013 cases of IBCs that were surgically resected from 1,006 patients were selected for this study.

The data including mean HER2 copy number, mean CEP17 copy number, HER2/CEP17 ratio, and the number of counted nuclei were obtained from the FISH reports. Polysomy 17 was defined as mean copy number of CEP17 $\geq$ 2.6, in accordance with the previous study by Tse et al (17). Although there are no standard criteria for polysomy 17, we defined the cutoff value for polysomy 17 as CEP1 $\geq$ 2.6 considering the possible truncation effect of FISH. Using this criterion, 300 from 1013 (29.6%) IBCs were designated as having polysomy 17.

2. Tissue microarray construction

Of the 300 cases with CEP17 ≥ 2.6, forty-seven cases with little residual tumor tissue were excluded, and the remaining 253 cases were used for construction of tissue microarray (TMA) blocks. To overcome sampling errors caused by TMA evaluation, all hematoxylin and eosin-stained slides and immunohistochemical stained slides for HER2 were reviewed, and sections most representative of the tumor were chosen for TMA construction. Three tissue columns of invasive carcinomas (2.0 mm in diameter) were taken from different areas of the tumors and arranged in new tissue microarray blocks using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). In each TMA block, two columns of normal breast tissue were included as negative control.

3. Immunohistochemistry of HER2

Expression of HER2 was re-evaluated on TMA sections. Four μm-thick tissue sections were cut, dried, deparaffinized, and rehydrated following standard procedures. All sections were subjected to heat-induced antigen retrieval. Immunohistochemical staining was carried out in a BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ, USA) using an i-View detection kit (Ventana Medical Systems) for HER2 (rabbit monoclonal; 4B5;

Ventana). According to 2007 ASCO/CAP guidelines, HER2 expression was scored as follows: 0, no staining; 1+, weak and incomplete membranous staining in 10% of the tumor cells; 2+, weak to moderate, complete membranous staining in 10% of the tumor cells; 3+, strong, complete membranous staining in 30% of the tumor cells.

4. Fluorescent in situ hybridization

To evaluate each copy number of SMS, HER2, CEP17, RARA, and TP53, we performed FISH on TMA slides using commercially available locus-specific probes and chromosome enumeration probe (CEP); SMS (17p11.2):RARA (17q21.2) and TP53 (17p13.1):CEP17 (Abbott Molecular, Downers Grove, IL, USA). HER2 FISH was performed using the PathVysion assay (Abbott Molecular).

Briefly, 4μm deparaffinized TMA sections were incubated in pretreatment solution (Abbott Molecular) at 80℃ for 30 minutes, then in protease solution (Abbott Molecular) for 20 minutes at 37℃. Probes were diluted in tDen-Hyb-2 hybridization buffer (InSitus Biotechnologies, Albuquerque, NM). Co-denaturation of the probes and DNA of the tissue sections was achieved by incubating for 5 minutes at 73℃ using a HYBriteTM (Abbott Molecular) followed by 16-hour hybridization at 37℃.

Post-hybridization washes were performed according to the protocols. Slides were mounted in 4',6-diamidino-2-phenylindole/ anti-fade and viewed with a fluorescence microscope. Gene signals per cell were evaluated in 50 tumor nuclei for each TMA core. Average gene copy number was calculated separately for 3 cores and the largest mean copy number among them was chosen for analysis.

5. Re-grading of HER2 status using additional reference genes

HER2 status was assessed with three different standard criteria. First, HER2 status was determined by 2007 ASCO/CAP guidelines using HER2:CEP17 ratio. HER2:CEP17 ratio of less than 1.8 was considered non-amplified, 1.8 to 2.2 was considered equivocal, and more than 2.2 was considered amplified. Second, it was classified by mean HER2 copy number using 2007 ASCO/CAP guidelines. Mean number of HER2 gene per cell less than 4 was considered non-amplified, 4 to 6 equivocal, and more than 6 was considered amplified (9). Third, we applied updated 2013 ASCO/CAP guidelines. HER2 copy number of 6.0 or higher per cell, or HER2/CEP17 ratio of 2 or higher was defined as HER2-positive. The cases with HER2/CEP17 ratios less than 2 but HER2 copy numbers of 4 or higher to less than 6 signals per cell were considered equivocal. HER2 copy numbers less than 4 signals per cell and HER2/CEP17 ratios less than 2 were defined as

negative (15).

Lastly, we introduced additional reference genes to evaluate HER2 status. If one or more of SMS, RARA, or TP53 signals were found to be less than 2.6 per cell, we assumed that such cases were not true polysomy 17. Any additional genes with ≥ 2.6 signals per cell were not considered appropriate as reference genes. Thus, the gene with the highest signal count among those with less than 2.6 signals per cell was selected as a new chromosome 17 reference gene instead of CEP17 for calculation of the HER2 reference gene ratio (17).

6. Array based comparative genomic hybridization

Genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tumor tissues in select 8 cases. As a reference, commercially available genomic DNA extracted from normal human frozen tissues (Macrogen, Seoul, Korea) was used. Breast cancer slides were reviewed and representative areas were marked on H&E slides. Then, tumor tissues were manually dissected under the microscope using three to five serial sections (4 μm thick). These tissues were subjected to tissue lysis using proteinase K lysis buffer containing 0.5% Tween 20 (Sigma, St. Louis, MO, USA), 100 mM of Tris HCl buffer (pH 7.6), 1 mM of EDTA, and 1 mg/ml of proteinase K (Sigma) at

55℃ for 24 h to 48 h. To validate the copy number variation of chromosome 17 in 8 cases, we performed array-based comparative genomic hybridization (aCGH) analysis of the tumor genome using a whole-genome platform, SurePrint G3 Human CGH 4x180K Microarray (Agilent, Inc., Santa Clara, CA, USA), including 170,182 oligonucleotides with approximately 13kb spacing. Among them, 5,237 oligonucleotides belonged to chromosome 17. The probe sequences were based on the human genome reference (hg19). Array CGH experiments were performed according to the manufacturer's instructions. Tumor and reference genomic DNAs (200ng) were digested with restriction enzymes and labeled with Cy3-deoxycytidine triphosphate (tumor) and Cy5-deoxycytidine triphosphate (reference) with the Agilent DNA Labeling Kit. Labeled tumor and reference DNAs were combined, denatured, pre-annealed and then hybridized to the arrays for 40 hours at 65℃. After hybridization and recommended washes, the arrays were scanned with an Agilent G4900DA Surescan Microarray scanner. Images were analyzed with Feature Extraction Software v11.0.1.1 (Agilent Technology) for background subtraction and lowness normalization.

Analyses of copy number variation were performed using Agilent Genomic Workbench v7.0.4.0 software (Agilent Technology). We used the Aberration Detection Method 2 (ADM2) statistical algorithm with threshold

6.0. A genomic segment was considered gain or loss when the log2 ratio of the tumor/reference fluorescent intensities of a given region encompassing at least three probes was > 0.25 or < −0.25, respectively. A genomic segment was considered as amplified when the log2 ratio of the tumor/reference fluorescent intensities of a given region encompassing at least three probes was >0.58. Oligonucleotide probes that belonged to genes are as follows: TP53 (2 probes: A_14_P104532 and A_14_P122951), SMS (12 probes: A_16_P20602682, A_16_P20602712, A_14_9117402, A_16_940769741, A_14_P127204, A_16_920602893, A_14_P107021, A_16_P03222877, A_16_p40769961, A_14_P130517, A_16_P03222912 and A_16_P03222921), HER2 (2 probes: A_14_P121276 and A_14_P114826) and RARA (4 probes: A_16_P03243741, A_14_P109913, A_14_P137156 and A_14_P103451). Because HER2 and TP53 had only 2 probes, we represented mean log2 ratio of each oligonucleotides probe comprising each gene to assign the copy number status of 4 genes. Chromosomal plots for each case were presented using Agilent Genomic Workbench v7.0.4.0 software (Agilent Technology).

7. Statistical analysis

After excluding cases in which FISH analysis failed for all genes (10 cases), a total of 243 IBCs were available for re-grading of HER2 status. FISH failures were due to detachment of the tissue core on TMA, lack of

tumor cells in the arrayed tissue, or inadequate hybridization. Statistical significance was analyzed using Statistical Package, SPSS version 21.0.0 for Windows (SPSS Inc, Chicago, IL, USA). Pearson's chi-square test was used to analyze the relationship between CEP17 copy number and HER2 status defined by different diagnostic criteria. The concordance rates between HER2 status and immunohistochemistry results of HER2 as well as between HER2 statuses assessed using different criteria were analyzed using kappa statistics. P values < 0.05 were considered statistically significant. All p values were two-sided.

RESULTS

1. HER2 status and CEP17 copy numbers in 1013 IBCs

The distributions of HER2 and CEP17 copy numbers in diagnostic FISH reports are shown in Figure 1. Mean HER2 and CEP17 copy numbers of 1013 IBCs ranged from 1.05 to 51.50 and from 1.10 to 14.95, respectively. Median values of mean HER2 and CEP17 copy numbers were 2.45 and 2.05. Based on the HER2:CEP17 ratio stated in 2007 ASCO/CAP guidelines, 207 (20.4%) of 1013 IBCs were HER2-amplified, 789 (77.9%) were non-amplified, and 17 (1.7%) were equivocal. Based on mean HER2 copy number, 207 (20.4%) had > 6 mean copy numbers of HER2, 77 (7.6%) had copy numbers between 4 and 6, and 729 (72.0%) had < 4 (Table 1).

Three hundred (29.6%) of 1013 cases showed ≥ 2.6 mean CEP17 copy numbers and were defined as polysomy 17 (Table 1). The frequency of polysomy 17 was higher in HER2 amplified or equivocal groups than in the non-amplified group using HER2:CEP17 ratio (54.1%, 47.1% and 22.8%, respectively; $p<0.001$). It was also higher in breast cancers with > 6 mean HER2 copy numbers or 4 to 6 copy numbers than in those with <4 copy numbers (61.4%, 72.7%, and 16.0%, respectively; $p<0.001$). In this polysomy 17 group, 183 (61.0%) of 300 had ≥ 4 HER2 copy numbers.

We also applied updated 2013 ASCO/CAP guidelines for assessment of HER status in breast cancers of our cases. The amplified cases were slightly increased (20.4% to 22.3%), but equivocal cases were significantly increased (1.7% to 6.0%), compared to those graded based on HER2/CEP17 ratio given by 2007 ASCO/CAP guidelines. Most (90.2%) of the equivocal cases showed increased CEP17 copy numbers.

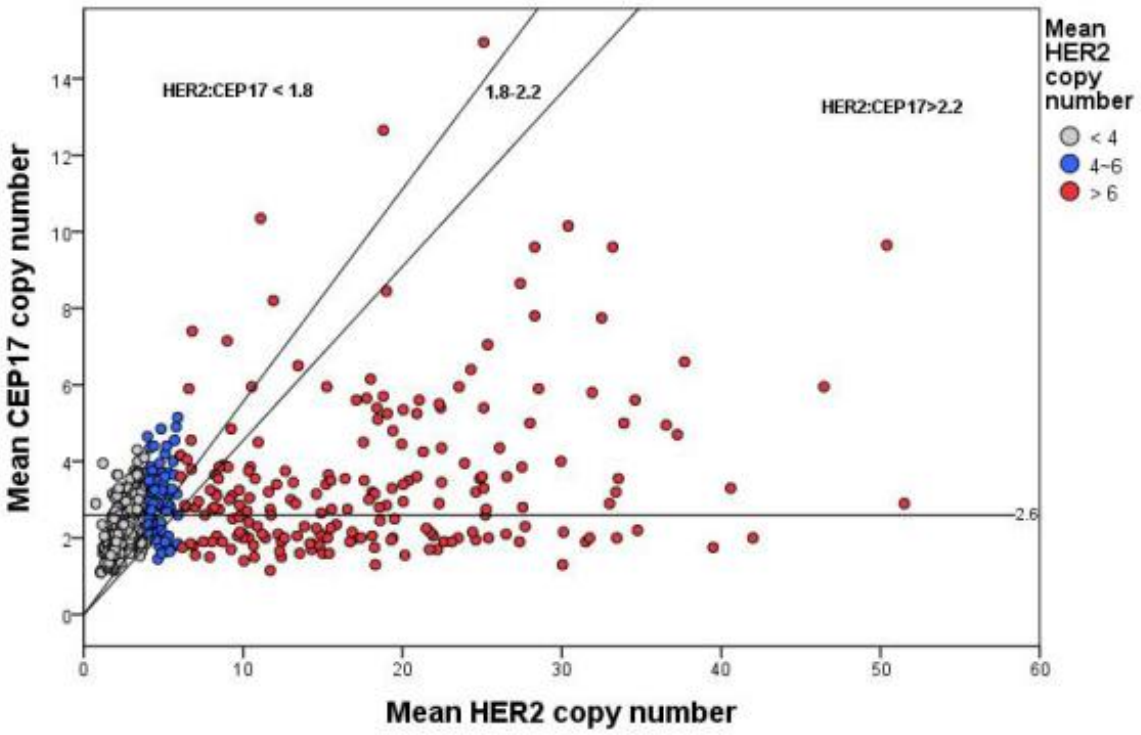


Figure 1. Distribution of mean HER2 and CEP17 copy numbers in 1013 invasive breast cancers. The cases (colored circles) are plotted based on the CEP17 copy number and HER-2 gene copy number. Vertical line indicates cutoff value for polysomy 17 (CEP17 copy numbers ≥ 2.6). Two tilted reference lines indicate HER2:CEP17 ratio of 1.8 (left sided) and 2.2 (right sided).

Table 1. Comparison of HER2 status and increased CEP17 copy number according to three different criteria in 1013 invasive breast cancers

Criteria	CEP17 < 2.6 (n=713)	CEP17 ≥ 2.6 (n=300)	P-value*
HER2 status by HER2/CEP17 ratio			
< 1.8 (n=789)	609 (77.2%)	180 (22.8%)	< 0.001
1.8-2.2 (n=17)	9 (52.9%)	8 (47.1%)	
> 2.2 (n=207)	95 (45.9%)	112 (54.1%)	
HER2 status by mean HER2 copy number			
< 4.0 (n=729)	612 (84.0%)	117 (16.0%)	< 0.001
4.0-6.0 (n=77)	21 (27.3%)	56 (72.7%)	
> 6.0 (n=207)	80 (38.6%)	127 (61.4%)	
HERs status by updated ASCO/CAP guidelines in 2013 †			
Non-amplified (n=726)	609 (83.9%)	117 (16.1%)	< 0.001
Equivocal (n=61)	6 (9.8%)	55 (90.2%)	
Amplified (n=226)	98 (43.4%)	128 (56.6%)	

*P-value was calculated using Pearson's chi-square test.

†HER2 amplification is defined as HER2 copy number of ≥ 6.0 signals per cell, or HER2/CEP17 ratio of ≥ 2. The equivocal category is defined as a ratio of < 2 but copy number of ≥ 4 to < 6 signals per cell. A copy number of < 4 signals per cell and ratio of < 2 are defined as HER2 non-amplification.

2. Additional FISH results using SMS, RARA and TP53 probes in 243 tissue microarray samples

The additional FISH results were shown in Table 2. All cases showed CEP 17 copy number of ≥ 2.6 in additional experiments. Among SMS, RARA, and TP53, TP53 showed least changes in the gene copy numbers. RARA revealed copy number gains most frequently. Fifty eight (23.9%) of 243 samples showed copy number gain of RARA (≥ 2.6 and < 6.0 copy numbers), and 12 (4.9%) revealed amplification (≥ 6 copy numbers). Compared to RARA, SMS and TP53 frequently showed copy number loss (< 1.6 copy numbers) (Figure 3). Seventy-seven (31.7%) of 243 samples had copy number loss of SMS, and 28 (11.5%) of TP53.

We used a web-based mining tool, "www.cbioportal.org", to obtain information on copy number variations of the genes by aCGH (36, 37). We used the dataset from The Cancer Genome Atlas (TCGA) containing 943 breast cancer cases (TCGA, Provisional, last cited of July, 2013) (38). Table 3 shows the copy number variations of 4 genes by aCGH from TCGA dataset. The general proportions of each group were similar to our results by FISH but losses of TP53 were more frequently observed.

On the basis of copy number alterations of HER2 and the 4 reference genes,

we could build the schematic presentation of distributions and extents of amplicons on chromosome 17 (Figure 4). In 2 of 243 samples, the copy numbers of all 5 probes showed ≥ 2.6 (Figure 5). We suspected that they had true polysomy 17. Other samples showed various patterns of copy number alterations. The most frequent pattern was that of a short amplicon including only HER2 and CEP17 areas (57.6%, 140/241). Most amplicons were placed in long arm of chromosome 17 and pericentromeric areas. Only 10 cases had large amplicons including both long and short arms.

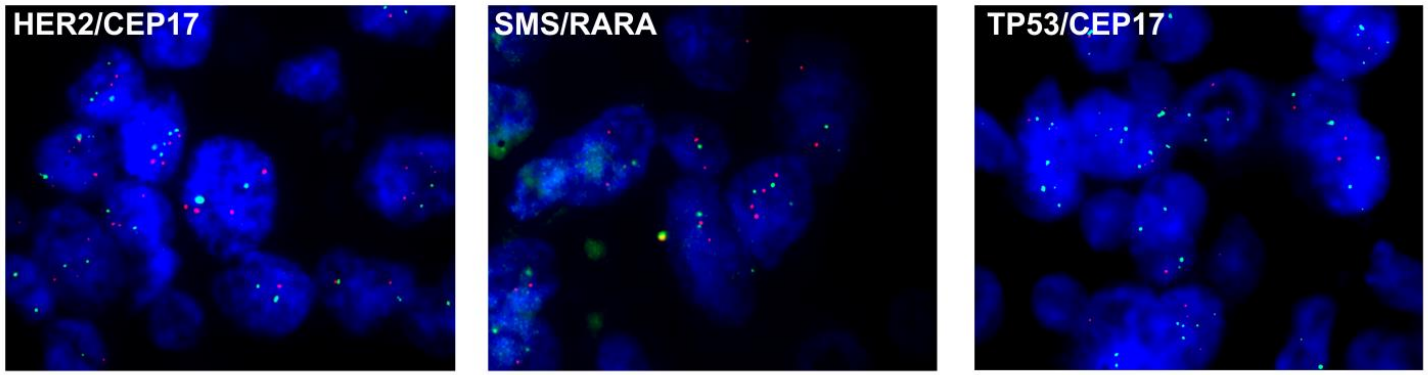


Figure 2. A representative example of copy number loss in TP53. Case #156 had increased CEP17 and HER2 copy numbers with mean copy number of 4.35 and 3.8, respectively. Copy numbers of SMS and RARA showed no alterations (mean copy number; SMS, 2.12; RARA, 2.04), but that of TP53 (red signal) was decreased (mean copy number, 1.52).

Table 2. Copy number variation of additional reference genes on chromosome 17 in 243 tissue microarray samples by FISH analyses

Copy number Status*	HER2	RARA	SMS	TP53
Copy number loss	1 (0.4%)	37 (15.2%)	77 (31.7%)	28 (11.5%)
Disomy	15 (6.2%)	136 (56.0%)	154 (63.4%)	190 (78.2%)
Copy number gain	133 (54.7%)	58 (23.9%)	11 (4.5%)	25 (10.3%)
Amplification	94 (38.7%)	12 (4.9%)	1 (0.4)	0 (0%)
Co-amplification with HER2†	-	10/94 (10.6%)	1/94 (1.1%)	0/94 (0%)

*Copy number loss, mean copy number < 1.6; disomy, 1.6 ≤ mean copy number < 2.6; copy number gain, 2.6 ≤ mean copy number ≤ 6.0; amplification, mean copy number > 6.0

†Of the 94 HER2 amplified cases (> 6.0 HER2 copy numbers), those with amplification in each gene (> 6.0 mean copy numbers) is presented.

Table 3. Copy number variation of 934 breast cancers by aCGH from TCGA dataset

Copy number status	HER2	RARA	SMS	TP53
Homozygous deletion	1 (0.1%)	2 (0.2%)	5 (0.5%)	10 (1.0%)
Heterozygous deletion	228 (24.4%)	264 (28.0%)	178 (18.9%)	561 (59.5%)
Diploid	405 (42.9%)	420 (44.5%)	587 (62.2%)	318 (33.7%)
Low level gain	179 (19.0%)	189 (20.0%)	149 (15.8%)	44 (4.7%)
High level amplification	121 (12.8%)	59 (6.3%)	15 (1.6%)	1 (0.1%)
Co-amplification with HER2*	-	59/121(48.8%)	4/121(3.3%)	0/121 (0%)

*Of the HER2 amplified cases, the number of cases with amplification in each gene is presented.

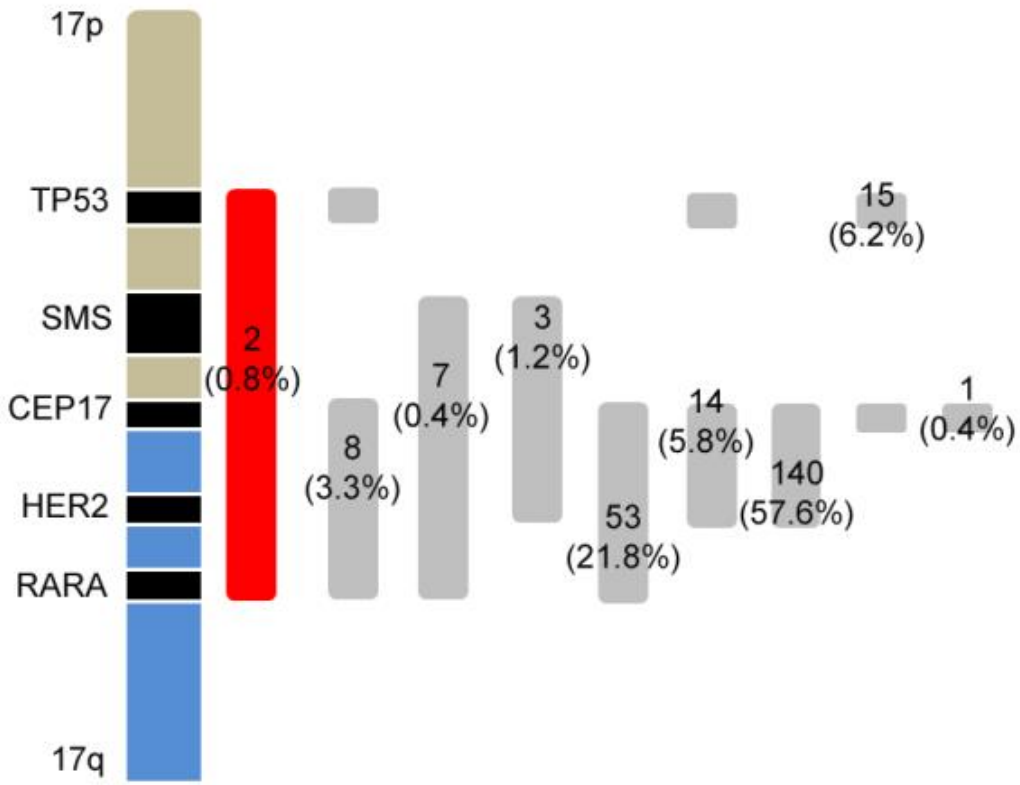


Figure 3. Schematic drawing of presumed extent of amplicons on chromosome 17 based on the results of FISH analyses using additional reference genes. The red bar indicates 2 possible true polysomy 17 cases. The numbers in the red and gray bars indicate the number of cases corresponding to each group

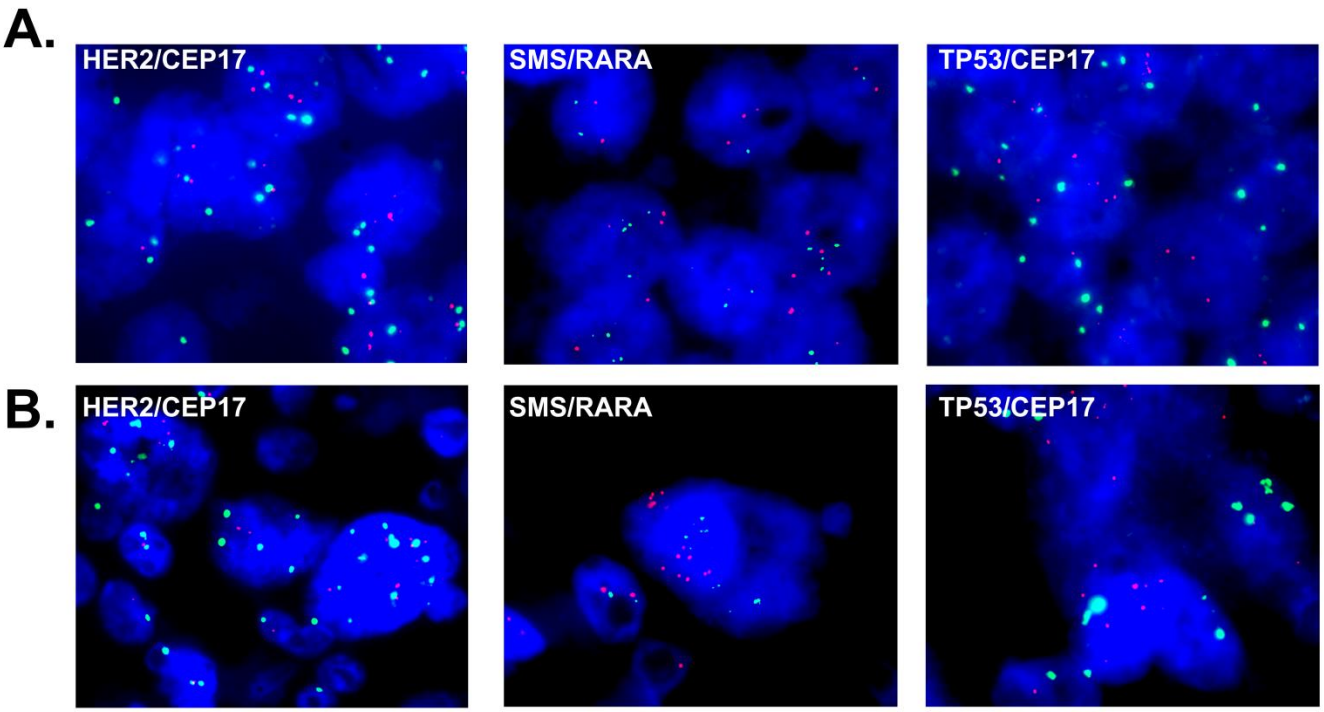


Figure 4. Two cases with suspected true polysomy 17 by FISH analyses. Representative photos of FISH using additional reference probes show increased copy numbers for all examined genes for case #155 (A) and case #250 (B).

3. Re-graded HER2 status after additional FISH analyses

After additional FISH studies, we re-graded the HER2 status based on the gene copy number of the additional reference gene. Two cases in which all the 5 probes had copy number of 2.6 and higher were excluded for re-grading. Table 4 details the HER2 status between different criteria. Among the cases with HER2:CEP17 ratio less than 1.8, HER2 status was upgraded to amplified in 42 cases (27.8%), and to equivocal in 33 (21.8%) of 151. Among the cases with HER2:CEP17 ratios between 1.8 and 2.2, 7 (87.5%) of 8 were upgraded to HER2-amplified. All cases which were diagnosed as amplified based on HER2:CEP17 ratio retained the same status after re-grading. In total, 82 (34.0%) of 241 cases were upgraded after additional FISH analyses. Using 2013 ASCO/CAP guidelines, only two among 101 non-amplified cases were upgraded to amplified, and 23.8% of non-amplified cases were reclassified as equivocal. In equivocal cases, 76.1% were reclassified as amplified. However, all amplified cases retained the same status.

We also investigated the relationship between HER2 copy numbers and changes of HER2 status after additional FISH studies (Table 4). When mean HER2 copy number was less than 4, 24 (23.8%) of 101 cases were upgraded from non-amplified to equivocal, and 2 (2.0%) cases were upgraded from non-amplified to amplified. When mean HER2 copy number was between 4

and 6, 36 (76.6%) of 47 cases were upgraded from equivocal to amplified and 1 (2.1%) case were downgraded from equivocal to non-amplified. All cases which were amplified by mean HER2 copy numbers (> 6) remained the same after re-grading.

Table 4. HER2 status by FISH analyses using additional reference genes on chromosome 17.

Criteria	Total	*HER2* status based on additional reference genes			Kappa coefficient*
		Non-amplified	Equivocal	Amplified	
HER2 status by *HER2*:CEP17 ratio					
Non-amplified (<1.8)	151	76 (50.4%)	33 (21.8%)	42 (27.8%)	0.445
Equivocal (1.8~2.2)	8[a]	0 (0%)	1 (12.5%)	7 (87.5%)	
Amplified (>2.2)	82[c]	0 (0%)	0 (0%)	82 (100%)	
HER2 status by mean *HER2* copy number					
Non-amplified (<4)	101	75 (74.3%)	24 (23.8%)	2 (2.0%)	0.585
Equivocal (4~6)	47	1 (2.1%)	10 (21.3%)	36 (76.6%)	
Amplified (>6)	93[d]	0 (0%)	0 (0%)	93 (100%)	
HER2 status by ASCO/CAP guidelines in 2013					
Non-amplified	101	75 (74.3%)	24 (23.8%)	2 (2.0%)	0.591
Equivocal	46	1 (2.1%)	10 (21.3%)	35 (76.1%)	
Amplified	94[e]	0 (0%)	0 (0%)	94 (100%)	
Total	241	76 (31.5%)	34 (14.1%)[b]	131 (54.4%)[f]	

*P-value is < 0.001

a vs. b, P<0.001; c vs. f, d vs. f, e vs. f, P<0.001 by Chi-square test

4. Correlation between re-graded HER2 status by additional reference genes and HER2 immunohistochemistry

The correlations between HER2 gene status by various criteria and HER2 IHC are shown in Table 6. Of the 253 cases with increased CEP17 copy numbers (≥2.6), more than half (55.7%) demonstrated negative (0~1+) immunostaining for HER2. Eighty-nine (35.2%) of the 253 cases showed positive (3+) immunostaining. Only 23 (9.1%) of 253 cases showed equivocal (2+) immunostaining. We evaluated the concordance rate between HER2 FISH results by standard methods based on ASCO/CAP guidelines and our new method employing additional reference genes and HER2 protein expression by IHC. We observed a good agreement ($\kappa = 0.624$, $p < 0.001$) between IHC result and conventional FISH results using HER2:CEP17 ratio by 2007 ASCO/CAP guidelines. When updated 2013 guidelines were applied, the kappa value became poorer ($\kappa = 0.485$) (Table 5). However, after re-grading by additional reference genes, cases with negative immunostaining were upgraded to amplified in substantial portions, and thus, the agreement between FISH and IHC results significantly declined ($\kappa = 0.390$, $p < 0.001$).

Table 5. Correlation between HER2 FISH and HER2 immunohistochemistry

Criteria		HER2 IHC			Kappa coefficient†
		Negative (0~1+)	Equivocal (2+)	Positive (3+)	
HER2 Status based on additional FISH analyses*	Non-amplified	66/76 (86.8%)	6/76 (7.9%)	4/76 (5.3%)	0.390
	Equivocal	30/34 (88.2%)	3/34 (8.8%)	1/34 (2.9%)	
	Amplified	37/131 (28.2%)	13/131 (9.9%)	81/131 (61.8%)	
	Total	133/241 (55.2%)	22/241 (9.1%)	86/241 (35.7%)	
HER2 Status based on ASCO/CAP guideline 2007 using *HER2*/CEP17 ratio	Non-amplified	130/161 (80.7%)	16/161 (9.9%)	15/161 (9.8%)	0.624
	Equivocal	3/8 (37.5%)	2/8 (25.0%)	3/8 (37.5%)	
	Amplified	8/82 (9.5%)	5/82 (6.0%)	71/82 (84.5%)	
	Total	141/253 (55.7%)	23/253 (9.1%)	89/253 (35.2%)	
HER2 Status based on ASCO/CAP guideline 2013	Non-amplified	90/105 (85.7%)	9/105 (8.6%)	6/105 (5.7%)	0.485
	Equivocal	37/49 (75.5%)	6/49 (12.2%)	6/49 (12.2%)	
	Amplified	14/99 (14.1%)	8/99 (8.1%)	77/99 (77.8%)	
	Total	141/253 (55.7%)	23/253 (9.1%)	89/253 (35.2%)	

*Re-grading by additional FISH analyses were available in 241 cases.

†P-value is < 0.001

5. Comparison between FISH and aCGH in 8 cases

We performed aCGH in select 8 cases to investigate the accuracy of FISH analyses with additional reference genes. Each case showed a different pattern of copy number variation by FISH analyses (Appendix A2). Table 6 shows comparison between the results of FISH and aCGH, and Figure 6 shows chromosomal plots for each case. Concordance between gene copy number evaluated by FISH and mean log2 ratio by aCGH was poor.

Two of 8 cases (#155 and # 250) were suspected to have true polysomy 17 by FISH analyses, but there was no copy number gain of whole chromosome by aCGH. Case #115 showed a copy number gain in chromosomal regions belonging to RARA and HER2, and amplification in TP53 gene based on the mean log2 ratio of the probes. In FISH analysis, mean copy number of SMS was 2.84 in #115, but there was no evidence of gain or amplification in SMS probes. #250 also showed mean copy number higher than four in RARA, HER2, SMS and TP53. However, it only showed gain of TP53 probes (Table 6; Figure 6 (E) and (G)). #115 and #250 were classified as non-amplified by conventional HER2:CEP17 FISH and finally identified as non-amplified by aCGH.

Five of the 8 cases (#15, #89, #104, #116 and #163) were upgraded from non-amplified to amplified after additional FISH studies (Table 6). Two (#116

and #163) of the five cases revealed amplification in HER2 gene by aCGH (Table 6; Figure 6 (D) and (F)). Case #116 displayed amplification in entire long arm of chromosome 17, and case #163 displayed amplification of 17q12 including HER2 and RARA. Case #89 and #155 displayed copy number gain of HER2 (Table 6). In case #15, mean HER2 copy number was less than 4 and mean copy number of RARA, an alternative reference gene, was 1.56. However, the signals by FISH, and the mean log2 ratio by aCGH were not well-correlated. Based on aCGH, #15 displayed copy number gain of RARA.

The remaining case #251 was upgraded from non-amplified to equivocal by additional FISH. Mean copy number of HER2 was 3.35 but that of alternative reference gene, TP53 was as low as 1.6. Finally, it was re-graded to equivocal by additional FISH studies. However, aCGH study revealed that HER2 and other 3 gene were within normal copy number.

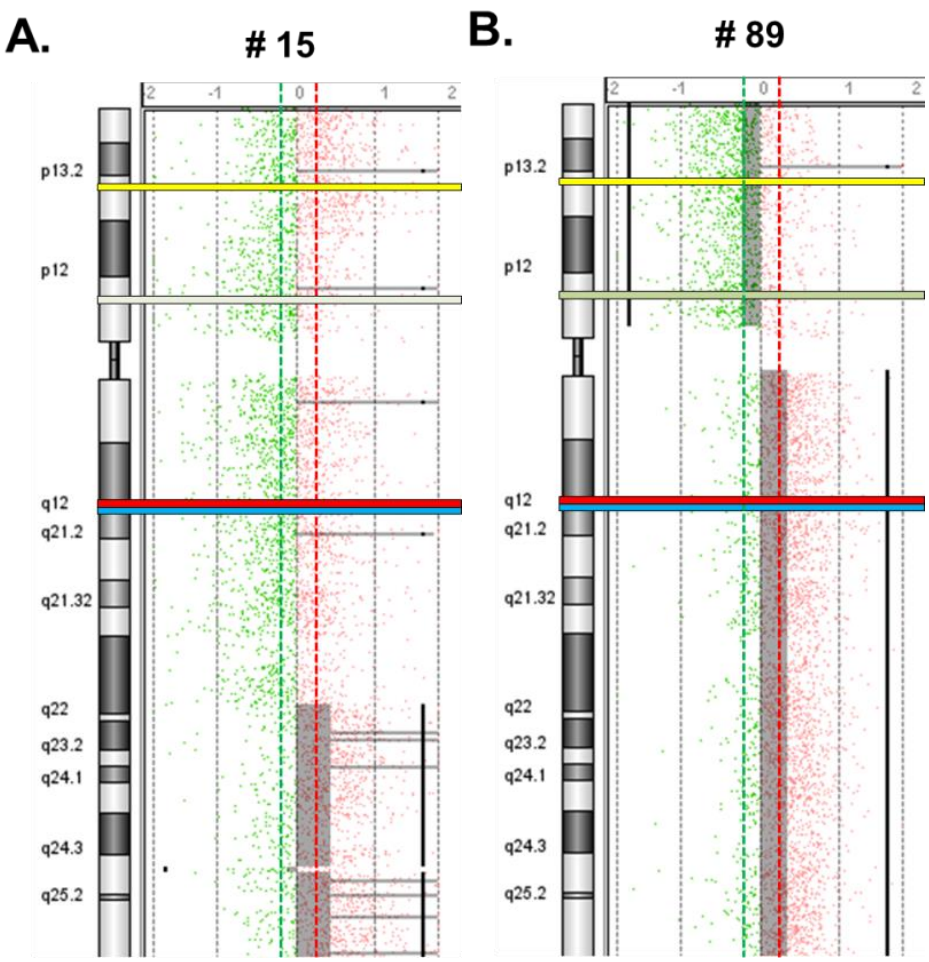
Table 6. Comparison between FISH and aCGH in the 8 selected cases

Case	Method	RARA	HER2	SMS	TP53	Interpretation
# 15	FISH*	1.56	3.55	1.48	4.46	HER2 non-amplified → amplified
	aCGH†	0.515	-0.31	-0.448	0.29	CNL of HER2; CNG of 17q21.2, 17q22 - 17q25.3, 17q23.2 and 17q25.1
	IHC		1+/3			No HER2 overexpression
# 89	FISH	1.88	4.3	2.6	1.92	HER2 non-amplified → amplified
	aCGH	0.346	0.346	-0.231	-0.231	CNG of HER2; gain of 17q
	IHC		1+/3			No HER2 overexpression
# 104	FISH	2.72	4.2	2.98	1.86	HER2 non-amplified → amplified
	aCGH	0.546	0.054	-0.462	0.532	No alteration of HER2; CNG of 17q21.2 and 17q23.2
	IHC		0/3			No HER2 overexpressed
# 116	FISH	3.14	5.1	1.94	2.8	HER2 non-amplified → amplified
	aCGH	0.657	0.657	0.262	0.262	Amplification of HER2; CNG of 17p and amplification of 17q
	IHC		1+/3			No HER2 overexpression
# 155	FISH	3.74	3.7	2.84	2.74	HER2 non-amplified → true polysomy 17 ?
	aCGH	0.252	0.367	-0.051	1.191	CNG of HER2; Multiple amplicons along 17q including 17q23.1 – 17q24.3
	IHC		3+/3			HER2 overexpression
# 163	FISH	6.8	5	1.68	1.82	HER2 non-amplified → amplified
	aCGH	0.723	1.107	-0.376	-0.376	Amplification of HER2; CNL of 17p and multiple small amplicons including 17q12 (HER2/RARA), 17q21.32 and 17q23.1 – 17q23.2
	IHC		3+/3			HER2 overexpression
# 250	FISH	5.6	6.75	4.1	5.2	HER2 non-amplified → true polysomy 17?
	aCGH	0.127	-0.251	-0.111	0.362	No alteration of HER2; amplification of p13.2, q23.2 and q25.3
	IHC		0/3			No HER2 overexpression
# 251	FISH	2.26	3.35	1.82	1.6	HER2 non-amplified → equivocal
	aCGH	0.205	0.084	-0.238	0.366	No alteration of HER2; Multiple small amplicons along the 17p and 17q
	IHC		2+/3			Equivocal of HER2 expression

*Mean copy number of each gene is presented.

†Mean log2 ratio of probes belonging to each gene is presented. If the gene is included in the copy number variation region defined by ADM2 algorithm, the mean log2 ratio of the genes belonging to those copy number variation regions are presented.

Abbreviations: CNG, copy number gain; CNL, copy number loss


A.
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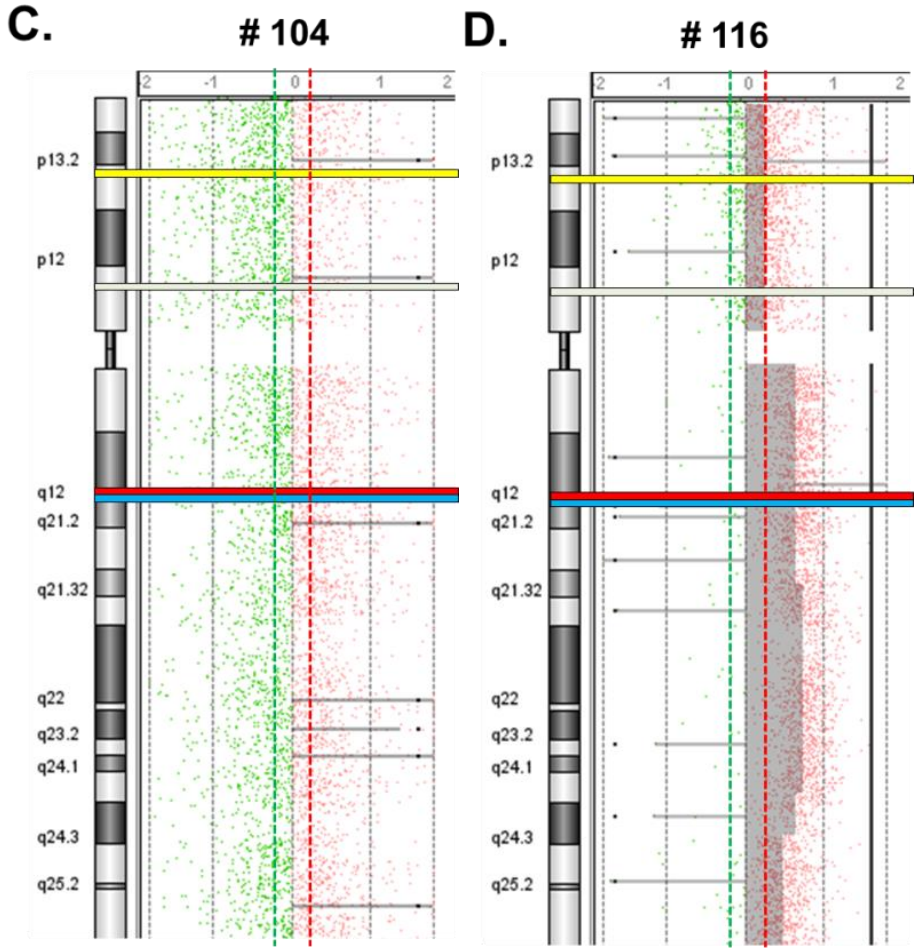
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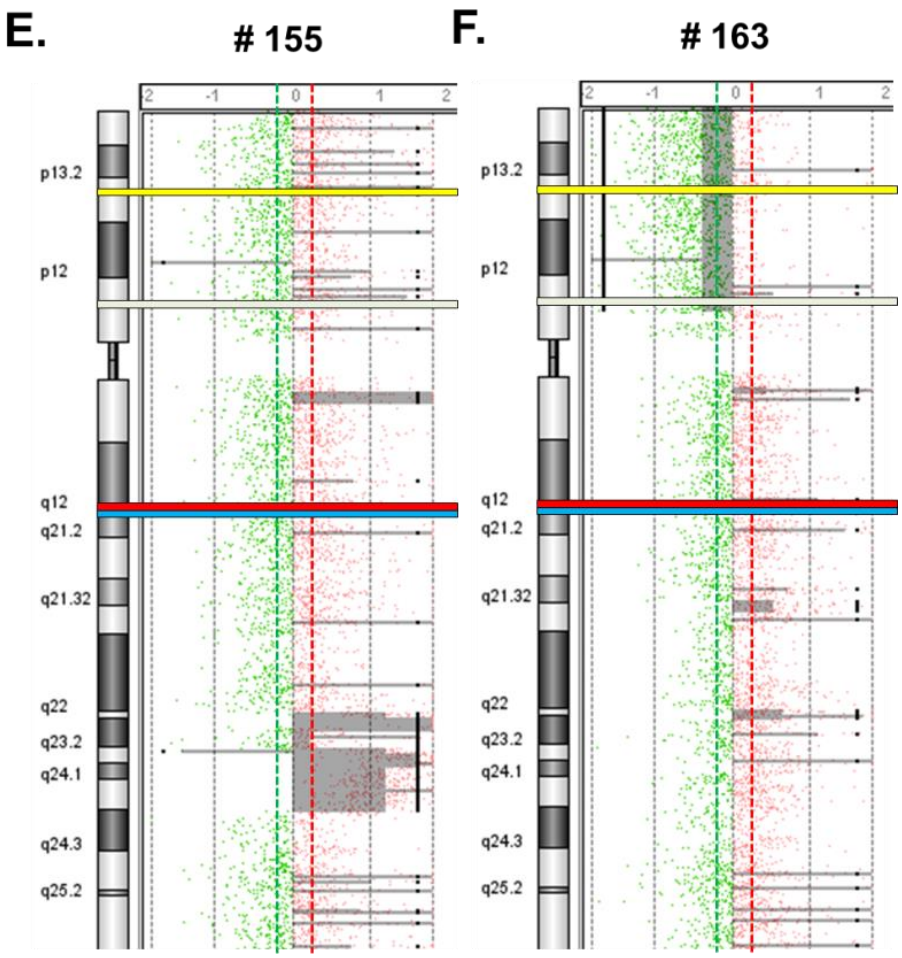
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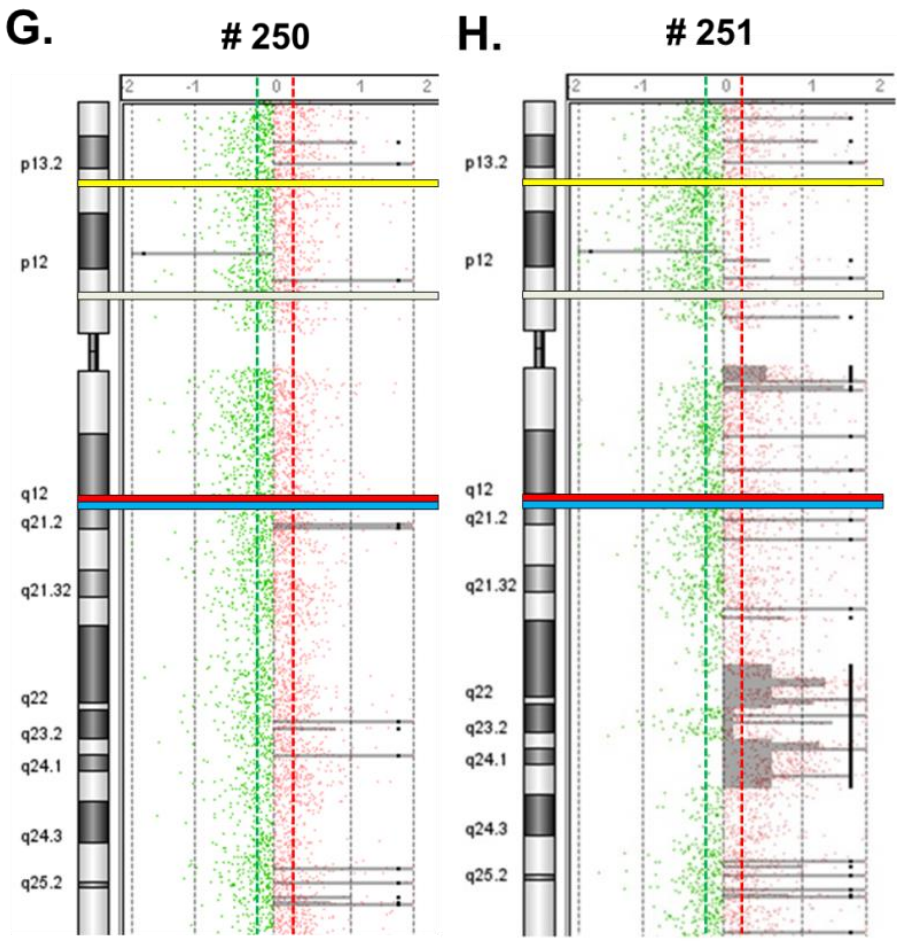


Figure 5. Chromosome plots of 8 cases by aCGH. Log 2 ratios of each oligonucleotide probe is plotted in red (above 0) and green (below 0) on the X-axis against each probe according to chromosomal location on the Y-axis; the genomic position of 4 genes are highlighted by colored boxes (yellow: TP53, green: SMS, red: HER2 and blue: RARA). Horizontal red and green dotted lines represent the cutoff between genomic gain and loss (log2 ratio >

0.25, log2 ratio < -0.25). Semi-transparent filled grey box and thick black vertical line represent copy number variation regions defined by ADM2 algorithm.

6. Pathologic review of cases with upgraded HER2 status after additional FISH analyses

We reviewed the pathologic characteristics of the 35 cases whose HER2 status were upgraded from equivocal (based on 2013 ASCO/CAP guidelines) to non-amplified after additional FISH studies. The results were demonstrated in Appendix A3. Most of the cases (30/35) were invasive ductal carcinomas, not otherwise specified (IDC, NOS) in histologic subtype. Two cases were invasive lobular carcinomas (ILC) and one was invasive micropapillary carcinoma. And the remaining two were metaplastic carcinomas. According to initial HER2 status, these two metaplastic carcinomas were classified as triple-negative breast cancer, but additional FISH results designated them as HER2+ subtype. In terms of histologic grade, there were two IDCs with low histologic grade. And about half (19/35) of the cases had low Ki67 proliferative index (< 20%).

Moreover, in these 35 cases, HER2 gene amplification was not accompanied by HER2 protein overexpression. Only 6 cases (17.1%) showed HER2 protein overexpression (3+). Five demonstrated equivocal (2+) immunoreactivity and the remaining 24 (68.6%) showed negative immunoreactivity (0 or 1+) to HER2.

To sum up, some pathologic features of the cases which were designated to have HER2 amplification after additional FISH were not compatible with those of HER2 amplified breast cancers.

DISCUSSION

Genome-based studies reveal that true polysomy is rare in breast cancers and most of cases, so-called polysomy 17 by ISH, are not a true increased number of whole chromosome but a gain of the pericentromeric or centromeric region, many experts concurred that polysomy 17 could affect the interpretation of results from ISH and lead to underestimation or overestimation of HER2 status. Recently updated 2013 ASCO/CAP guidelines finally focused on this issue and proposed options to avoid such false negative results of polysomy 17. One of such options is to repeat HER2 testing using another gene in chromosome 17 which is not expected to co-amplify with HER2. They recommended such re-testing when HER2:CEP17 ratio is < 2.0, average HER2 copy number is ≥ 4 and < 6 (equivocal group), and average CEP17 copy number is > 2.0 (15, 39).

We applied this method to 243 IBCs with increased CEP17 copy number (≥ 2.6) identified by FISH analyses in our institution. Tse and his colleagues also used this method in a relatively large series of 171 IBCs with polysomy 17, defined ≥ 2.6 CEP17 (17). In our and their studies, SMS, RARA and TP53 were used as additional reference genes, based on the concept that all of the genes in chromosome 17 would increase in true polysomy 17 and

any gene that showing eusomy in chromosome 17 could be used as a new surrogate instead of CEP17. Tse and his colleagues revealed that polysomy 17 was very rare (14%) by FISH analyses, and nearly half of the patients with an equivocal range of HER2 gene copy numbers and increased CEP 17 signals had their HER2 status upgraded from non-amplified to amplified using this additional verification method. Comparing the results from this study, our study, based on the same method, showed that only 2 cases would be possible true polysomy 17 by additional FISH analyses, and more than half (63.85%, 30/47) of cases with equivocal HER2 copy number were upgraded from non-amplified to amplified after additional analysis. Use of alternative genes as a new surrogate seemed to be helpful in finding hidden HER2-amplified cases within breast cancers with polysomy 17.

Although updated 2013 ASCO/CAP guidelines proposed a possible solution for the dilemma regarding polysomy 17, they did not provide specifics. As we mentioned earlier, updated guidelines simply proposed using other genes on chromosome 17 that are not expected to co-amplify with HER2 should be used. If so, which gene should we select from the numerous genes on chromosome 17? How many new genes do we have to use? What should we do if the HER2 status remained equivocal after alternative FISH? Unfortunately, we were not able to find answers to these questions but our

study did reveal some hints to these questions. The most important consideration that must be given upon choosing a surrogate for a chromosome is the stability of genomic copy number. If the gene we chose did not display diploidy, the status of HER2 would be false-negative or false-positive just as with using CEP17. RARA, SMS and TP53 have been used in previous studies. Because they are commercially available, they can be easily purchased and used for additional FISH analysis in the clinical setting. However, there was no evidence that they were suitable for reference of chromosome 17.

To evaluate the utility of RARA, SMS and TP53 as references, we assessed the copy number variation of these genes in breast cancers. According to copy number variation from TCGA data and our FISH results, we can evaluate the utility of 3 genes as surrogates of chromosome 17. First, RARA is a gene encoding retinoic acid receptorα (RARα) and located at 17q21 (40, 41). It maps very close to HER2 gene (0.65Mb) and is frequently amplified in HER2 amplified breast cancers. RARA is one of the genes that belong to a short HER2 amplicon which spans between 280 and 746kb (40). Paroni et al. demonstrated that 23–32% of HER2 amplified breast cancers showed co-amplification of RARA using quantitative PCR (41). Results from TCGA also demonstrate that 12.8% of breast cancers showed co-amplification of HER2 and RARA, and that 48.8% of HER2 amplified breast cancers

showed RARA amplification (Table 3). Our study showed that approximately 25% of breast cancers with increased CEP17 copy number showed copy number gain of RARA with HER2, and about 10% showed co-amplification of two genes by FISH (Figure 3 and Table 2).

Second, SMS also known as RAI1 (retinoic acid induced 1), is located on 17p11.2, and its chromosomal deletion is related to many congenital anomalies (43). The gene has been so barely studied in breast cancer that its copy number variation in breast cancer is not well-known. Based on aCGH results from TCGA dataset, only 1.3% of breast cancer showed high level amplification of SMS, and co-amplification of HER2 and SMS was identified in 3.3% of HER2 amplified breast cancers. However, gene loss was frequently identified. About 20% of breast cancers showed a low-level copy number loss which was regarded as heterozygote deletion by aCGH study (Table 6). In our study, 37.1% of the cases demonstrated decreased copy number of SMS. Such decreased copy numbers of SMS can result in overestimation of HER2 status. Marchio and his colleagues also showed that using SMS as reference gene could overestimate HER2 amplification for this reason, and the value of SMS as reference gene was limited in certain conditions (33).

Lastly, TP53 is a well-known tumor suppressor gene. It is located at 17p13.1, relatively far from HER2, and the possibility of sharing amplicon

between HER2 and TP53 is theoretically very low. TP53 is one of the most frequently mutated genes in breast cancer but copy number gain or amplification of TP53 is almost absent (42). However, chromosome 17p where TP53 is located is mainly involved in genetic losses in breast cancers (43). Even though our FISH studies showed that loss of TP53 (11.7%) was not common and that TP53 was the most stable gene that had least copy number alterations (Table 2), aCGH results from TCGA dataset show the frequency of genetic loss of TP53 is the most common among the three reference genes. Thus, TP53 also has limitations for use as surrogate.

Our study demonstrates that the majority of breast cancers with increased CEP17 copy number could show copy number changes of RARA, SMS and TP53 by FISH. Using any of these 3 genes can produce wrong interpretations when used alone. However, when the 3 genes are used altogether, most (94.2%, 227/241) of the cases had at least one gene with disomy in our study. Therefore, it is recommended to use a couple of genes together for accurate estimation of HER2 in breast cancers with increased CEP17 copy numbers. In practice, performing a couple of FISH analyses for diagnosis of one case may be a burden in terms of cost and time. To simplify application of this method and decrease costs, more studies will be needed in search of a gene on chromosome 17 that barely shows copy number alteration in breast cancer.

However, our study which used three alternative genes in combination still showed some problems. First, after re-grading using additional reference genes, HER2 equivocal cases were significantly increased compared to those based on 2007 ASCO/CAP guidelines. Moreover, 27% (36/131) of the amplified cases had HER2 copies between 4 and 6 (Table 4). These cases may have low levels of amplification of HER2 or no amplification at all. However, there is no way to establish the accuracy of HER2 status in this subset.

Another problem is the decreased concordance rate between HER2 FISH and IHC. Concordance between FISH and IHC became poorer after using additional genes. This discordance might be due to the increase in false-positive cases. After re-grading, about 30% of breast cancers with 0 or 1+ immunoreactivity were classified as amplified. They might be overestimated due to decreased copy number of the new reference gene.

The last is that the cases whose HER2 statuses were upgraded to be amplified by additional FISH studies were not compatible with HER2 amplified breast cancers in pathologic findings. Of the 35 cases with upgraded HER2 status from equivocal to amplified based on 2013 ASCO/CAP guideline, two cases were histologically metaplastic carcinomas, which are usually triple negative breast cancers. Moreover, in these cases, HER2 gene amplification was not accompanied by HER2 protein overexpression. Based

on these findings, SMS, TP53 and RARA do not seem to be appropriate alternative for CEP17, even if used in combination.

To confirm the accuracy of FISH analyses, we performed aCGH in 8 cases. There were some limitations in aCGH that we performed. First, we performed aCGH in a limited number of cases. Second, the aCGH platform that we used did not contain a centromeric region, and thus we could not confirm the true status of the centromere. Finally, we used formalin-fixed tissue for aCGH. Though many studies revealed that aCGH using FFPE was a reliable tool, there still exist chances for errors.

In spite of these limitations, we could obtain a few important findings from the results of aCGH. First, the results of FISH and aCGH were not well-correlated. Five of 8 cases were upgraded from non-amplified to amplified by additional FISH. Among them, only 4 cases were identified to have a gain of HER2 by aCGH. Moreover, case #15 and #250 that showed copy number gains of HER2 by FISH turned out to be loss by aCGH. A small number of HER2 probes that were contained in aCGH could be the reason for the discrepancy. There were only 2 probes that covered HER2 region and neither of the probes covered the entire region of HER2. If we used multiple probes that covered the entire gene, the results of aCGH could be different. Also, the intratumoral heterogeneity of tumor can be another reason. As the 8 cases

were selected after additional FISH analyses, the tumor areas examined in FISH and aCGH were not the same.

Second, we demonstrated that deducing the status of chromosome 17 by FISH of several genes could be incorrect. We made schematic pictures of chromosome 17 based on copy number alteration of 5 genes on it. There were 2 cases of possible true polysomy 17 in the schematic picture. The status of chromosome 17 in both cases turned out to be disomy after performing aCGH. They showed many small-sized amplicons throughout the whole chromosome, but not polysomy 17 (appendix A2). In conclusion, FISH does not seem to be an appropriate method for evaluation of chromosomal copy number status in breast cancers with increased CEP17 copy numbers.

CONCLUSION

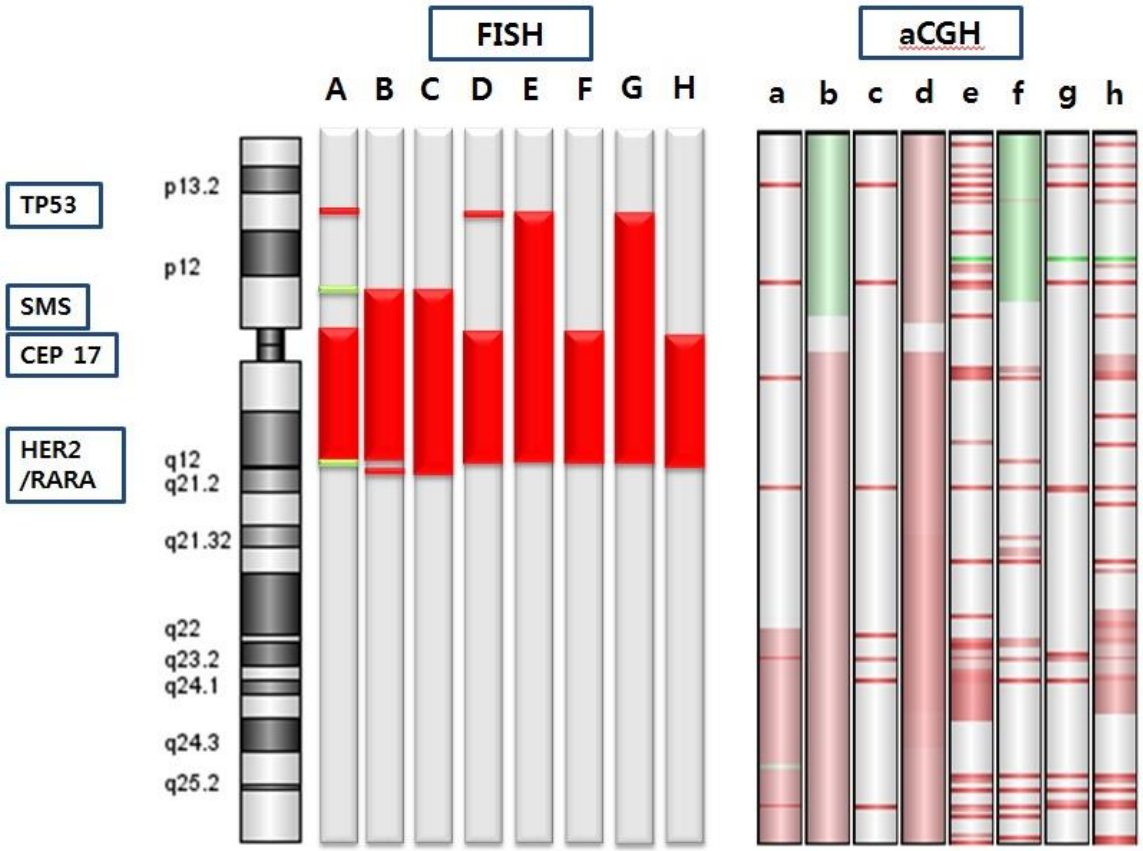
In conclusion, our study showed that using other reference genes on chromosome 17 as new surrogates for chromosome 17 status may be a way to evaluate HER2 status in breast cancers with increased CEP17 copy number. However SMS, RARA, and TP53 that we used in this study were not suitable as an independent marker. Combined use of these reference genes seemed to compensate the unstable copy number of each alternative gene. However, combined method still has some limitations. Performing FISH using multiple probes together is neither a simple nor affordable way in everyday practice. Also, this method can lead to over-grading of HER2 status when the tumor has loss of the new reference genes. Moreover, the accuracy of this method has not been verified thoroughly. It would be impossible to predict the complex genomic status by additional FISH analyses. Until now, three alternative genes, SMS, RARA and TP53 are not good choice for HER2 analysis. To apply this method in daily practice, further studies to find the most stable genes that barely show copy number alterations will be needed.

APPENDICES

A1. Log2 ratio of each probe that belongs to the region of TP53, SMS, HER2 and RARA

Gene	Name of probe.	Start	End	# 15	# 89	# 104	# 116	# 155	# 163	# 250	# 251
TP53	A_14_P104532	7572175	7572234	0.5404	0.5015	1.0614	0.2144	2.0005	0.4620	0.3641	0.6441
TP53	A_14_P122951	7576766	7576822	0.0388	-0.5096	0.0029	-0.0254	0.3817	0.0542	0.3606	0.0885
Average log2 ratio of TP53				0.2896	-0.0040	0.5321	0.0945	1.1911	0.2581	0.3624	0.3663
SMS	A_16_P20602682	17599791	17599850	-1.7041	0.1136	-1.3075	-0.4208	0.4665	0.0301	-0.0660	-0.2103
SMS	A_16_P20602712	17608564	17608623	-1.5061	0.5587	0.6113	-0.2542	-1.0204	-0.3923	0.1775	0.0666
SMS	A_14_9117402	17621582	17621641	0.1424	-0.7197	-1.5871	-0.1394	-0.4129	0.1289	0.1987	-0.3232
SMS	A_16_940769741	17630064	17630123	-0.2230	-0.5596	-1.0407	0.0241	0.4255	0.1552	-0.0546	-0.6868
SMS	A_14_P127204	17651916	17651975	0.3839	-1.0685	-0.1940	-0.4019	-0.1194	0.2083	-0.6329	0.0462
SMS	A_16_920602893	17666376	17666435	-1.0478	-0.3727	-0.0548	-0.2751	0.5365	-0.5315	0.0780	0.2819
SMS	A_14_P107021	17681968	17682013	-0.5503	-0.0580	-0.6386	0.3434	-0.3469	-0.4709	0.1764	-0.2655
SMS	A_16_P03222877	17691726	17691785	0.0171	-0.0731	0.1155	-0.3950	0.3935	-0.1852	0.4204	-0.1589
SMS	A_16_P40769961	17700447	17700497	-0.2668	-0.0676	-0.1182	-0.0927	-0.4169	-0.3171	-0.6266	-0.4941
SMS	A_14_P130517	17705510	17705561	-0.5650	-0.0125	-0.3799	0.2532	-0.4182	-0.4281	-0.2193	-0.3556

Gene	probe no.	Start	End	# 15	# 89	# 104	# 116	# 155	# 163	# 250	# 251
SMS	A_16_P03222912	17709572	17709631	-0.4952	-0.5827	-0.9551	0.5152	-0.3450	-0.3955	-0.2640	-0.6694
SMS	A_16_P03222921	17714588	17714647	0.4396	-0.9420	0.0020	-0.4177	0.6447	0.3919	-0.5174	-0.0917
Average log2 ratio of SMS				-0.4479	-0.3153	-0.4623	-0.1051	-0.0511	-0.1505	-0.1108	-0.2384
HER2	A_14_P121276	37856817	37856872	-0.9005	0.0584	0.4819	-0.0214	0.2735	1.8909	-0.1457	0.2913
HER2	A_14_P114826	37868158	37868207	0.2803	0.3757	-0.3738	0.1112	0.4606	1.5545	-0.3568	-0.1232
Average log2 ratio of HER2				-0.3101	0.2170	0.0540	0.0449	0.3671	1.7227	-0.2513	0.0840
RARA	A_16_P03243741	38484270	38484329	0.1235	0.1828	0.3937	0.1416	-0.1938	0.7608	0.1184	-0.1126
RARA	A_14_P109913	38493850	38493909	0.2936	0.4081	-0.0489	-0.0146	-0.0158	0.6384	-0.0199	0.0424
RARA	A_14_P137156	38507399	38507455	1.2311	0.6671	1.6703	0.1387	1.6009	1.0336	0.8599	0.9460
RARA	A_14_P103451	38509280	38509333	0.4135	0.2467	0.1691	0.2607	-0.3843	0.4587	-0.4513	-0.0567
Average log2 ratio of RARA				0.5154	0.3762	0.5460	0.1316	0.2518	0.7229	0.1268	0.2048


FISH
aCGH
A B C D E F G H
a b c d e f g h
TP53
SMS
CEP 17
HER2
/RARA
p13.2
p12
q12
q21.2
q21.32
q22
q23.2
q24.1
q24.3
q25.2

A2. Graphic illustration of chromosome 17 based on FISH and aCGH in 8 cases. Colored boxes that occupy each chromosome indicates copy number variation region by FISH and aCGH. Red boxes indicate amplification regions and green boxes indicate deletion regions.

A3. Pathologic characteristics of 35 cases with upgraded HER2 status from equivocal to amplified after additional FISH analyses based on 2013 ASCO/CAP guidelines

No.	Histologic subtype	Histologic grade	ER status	PR status	Molecular subtype*	Ki-67 index**	Mean HER2	Mean CEP17	HER2: CEP17 ratio	HER2 IHC
TA1801- 23	IDC	III	N	N	TNBC	High	4.25	3.20	1.33	0
TA1801- 28	IDC	II	P	P	Luminal A	Low	4.15	4.35	0.95	2+
TA1801- 40	IDC	I	P	P	Luminal B	High	4.50	3.65	1.23	0
TA1801- 46	IDC	III	N	N	TNBC	High	4.30	3.35	1.28	0
TA1801- 47	MPLC	III	N	N	TNBC	High	4.95	3.55	1.39	0
TA1801- 48	IDC	III	P	P	Luminal B	High	4.90	3.70	1.32	1+
TA1802- 09	MPLC	II	N	N	TNBC	High	5.25	3.15	1.67	1+
TA1802- 18	ILC	II	P	P	Luminal A	Low	4.40	4.40	1.00	1+
TA1802- 28	ILC	II	P	P	Luminal A	Low	4.15	3.40	1.22	0
TA1802- 32	IDC	I	P	P	Luminal A	Low	4.30	3.75	1.15	1+
TA1802- 35	IDC	III	P	P	Luminal B	High	4.85	3.40	1.43	0
TA1802- 44	IDC	III	N	N	TNBC	High	4.70	3.55	1.32	0
TA1802- 48	IDC	I	P	P	Luminal A	Low	4.20	2.80	1.50	0
TA1802- 50	IDC	II	P	P	Luminal A	Low	4.25	3.45	1.23	3+
TA1802- 54	IDC	II	P	P	Luminal A	Low	4.25	3.55	1.20	1+

TA1802- 55	IDC	II	P	P	Luminal A	Low	5.70	4.55	1.25	2+
TA1803- 03	IDC	III	N	N	TNBC	High	5.10	3.23	1.58	1+
TA1803- 12	IDC	III	P	P	Luminal B	High	5.80	4.90	1.18	1+
TA1803- 13	IDC	II	P	P	Luminal A	Low	4.75	3.95	1.20	1+
TA1803- 19	IDC	II	P	P	Luminal A	Low	5.42	2.78	1.95	3+
TA1803- 29	IDC	II	P	P	Luminal A	Low	5.83	3.15	1.85	1+
TA1803- 31	IDC	II	P	P	Luminal A	Low	4.70	3.50	1.34	0
TA1803- 32	IDC	II	P	P	Luminal A	Low	5.15	2.78	1.85	2+
TA1803- 40	IDC	III	N	N	TNBC	High	4.00	3.45	1.16	2+
TA1803- 42	IDC	II	P	P	Luminal A	Low	5.40	2.83	1.91	1+
TA1803- 50	IDC	III	N	N	TNBC	High	5.18	2.70	1.92	0
TA1803- 51	IDC	II	P	P	Luminal B	Low	5.00	3.40	1.47	3+
TA1803- 52	MPC	II	P	P	Luminal A	Low	5.10	3.60	1.42	1+
TA1804- 17	IDC	III	P	P	Luminal A	Low	4.35	2.90	1.50	2+
TA1804- 34	IDC	III	N	N	TNBC	High	5.65	3.65	1.55	3+
TA1804- 38	IDC	III	P	P	Luminal A	Low	4.45	3.25	1.37	0
TA1804- 47	IDC	III	N	N	TNBC	High	4.90	3.25	1.51	1+
TA1804- 50	IDC	III	P	P	Luminal B	High	5.20	4.40	1.18	3+
TA1805- 08	IDC	III	P	P	Luminal B	High	4.25	2.73	1.56	1+
TA1805- 15	IDC	III	P	P	Luminal A	Low	5.90	5.15	1.15	3+

Abbreviations: IDC, invasive ductal carcinoma, not otherwise specified; ILC, invasive lobular carcinoma; MPLC, metaplastic carcinoma; MPC, micropapillary carcinoma; TNBC, triple-negative breast cancer; IHC, immunohistochemistry; N, negative; P, positive

*Molecular subtype is based on initial HER2 status by HER2:CEP17 ratio by 2007 ASCO/CAP guideline.

**For Ki-67index, cases with 20% or more positive tumor cells were regarded as having a high proliferation index.

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국문 초록

서론: 일부 침윤성 유방암에서는 HER2 유전자의 평가를 위해 시행하는 제자리보합법 상, 17번 염색체 동원체의 복제 수(copy number) 증가가 비교적 흔히 관찰된다. 그러나 최근 연구에 의하면 유방암에서 17번 염색체의 다염색체성 (polysomy)은 매우 드문 현상이며, 제자리보합법 상 관찰되는 chromosome enumeration probe 17 (CEP17) 복제수의 증가는 동원체나 동원체 주변 구역의 유전적 증폭에 의한 것으로 밝혀졌다. 이러한 상황에서 CEP17을 17번 염색체의 수적 변화를 관찰하는 대리자로 사용하는 것은 한계가 있으며, 보다 정확한 HER2 유전자를 평가하기 위한 새로운 방법이 필요하다. 17번 염색체상에 있는 타 유전자를 CEP17을 대신하여 이용하는 방법은 이전 여러 연구들에서 제안된 바 있으며, 2013년 새로 제시된 ASCO/CAP 가이드라인에서도 언급되고 있다. 따라서 본 연구에서는 형광제자리보합법 상 CEP17의 복제수의 증가를 보이는 침윤성 유방암에 이 방법을 적용해 HER2 유전자의 수적 상태를 평가하고, 그 결과를 기존에 사용되고 있는 2007년과 2013년 ASCO/CAP 가이드라인을

토대로 한 결과와 비교해 보았다.

방법: 분당서울대학교병원에서 2004년 6월부터 2011년 11월까지 보고된 HER2 유전자의 형광제자리보합법 결과 보고서를 살펴본 후 총 300 증례의 CEP17 복제수의 증가를 보이는 증례를 발견하였다. 이 중 tissue microarray의 제작이 가능한 253 건의 증례를 최종 실험 대상으로 선정하여, RARA, SMS, TP53 유전자의 형광제자리보합법 검사를 시행하였다. 그 결과를 토대로 세가지 유전자의 복제수 중 최소 하나라도 2.6 미만의 평균 복제수를 보일 경우, 그 중 가장 큰 복제수를 보이는 유전자를 CEP17을 대신하여 HER2와의 비를 구하는데 이용하였고, 그러한 비를 기준으로 HER2 유전자의 상태를 평가하였다. 형광제자리보합법 검사 이후 8개의 대표적인 증례를 선정하여 고해상도 array 기반 비교유전체보합법을 시행, 재평가된 HER2의 수적 상태와 비교하여 그 정확도를 평가해 보았다.

결과: CEP17의 복제수가 2.6 이상인 243례의 유방암 중 2개의 증례에서 추가로 시행한 RARA, SMS, TP53 유전자와 HER2 유전자의 복제수가 모두 2.6 이상을 나타내었다. 2007년 가이드

라인을 기준으로 증폭을 보이지 않는다고 판단된 (HER2:CEP17<1.8) 증례 중 27.8%인 42 개의 증례가 추가적인 형광제자리보합법 검사 이후 HER2의 증폭을 보이는 것으로 변경되었다. 같은 방법을 2013년 가이드라인과 비교해 보았을 때 HER2의 증폭을 보이지 않는다고 판단되던 (HER2:CEP17<2.0) 101례의 중 2례가 증폭을 보이는 것으로, 24례가 HER2 상태 불명확 (equivocal)로 그 결과가 상향되었다. 또한 2013년 가이드라인에 의해 불명확 (equivocal)로 판단되던 46례 중 76.1%인 35례가 HER2 유전자의 증폭이 있는 것으로 상향되었다. 종합해보면 추가적인 형광제자리보법 검사 이후 HER2 유전자의 증폭을 보이는 것으로 분류된 증례가 유의하게 증가하였다. 그러나 형광제자리보합법과 면역조직화학염색간의 일치도는 오히려 감소하였다. Array를 바탕으로 하는 유전체비교보합법의 대상이 되는 총 8개의 증례 중 6례가 추가적 형광제자리보법 검사에 의해 HER2의 증폭을 보이는 것으로 상향 조정된 증례였다. 그러나 이 중 3례만이 실제로 HER2 유전자의 수적 증가를 보였다. 형광제자리보법상 실제 17번 염색체의 다염색성을 가진다고 생각되었던 2증례가 유전체비교보법상 실제 17번 염색체의

다염색성이 없는 것으로 밝혀졌다. 또한 새로운 방법에 의해 HER2의 결과가 상향된 증례들의 병리학적 특성을 살펴보았을 시, 다수의 증례에서 HER2 증폭을 보이는 유방암이 가지는 일반적 특성과는 불일치한 병리 소견을 보였다.

결론: 17번 염색체에 존재하는 다수의 유전자를 추가적인 표지자로 이용하여 HER2 유전자를 평가하는 것은 CEP17의 수적 증가를 보이는 유방암에서 기존의 방법에 비해 HER2를 보다 정확히 평가하는 대안 중 하나일 수 있다. 그러나 이 방법은 여러 한계점이 있다. CEP17을 대신할 유전자에 수적감소가 동반되어 있을 때, HER2 유전자 수적 상태가 과대평가된다. 본 연구에서 사용된 SMS, RARA, TP53의 경우 이러한 까닭으로 CEP17의 대체 표지자로 독립적으로 사용하기에는 부적절하였다. 또한 연구 결과 대체 표지자를 사용하는 방법은 Array기반의 비교유전체보합법의 결과와도 불일치를 보였다. 따라서 임상 진단에 SMS, RARA, TP35을 CEP17의 대리자로 사용하는 방법을 적용하는 것은 적절하지 않은 것으로 생각된다. 추가적 유전자를 통한 형광제자리보법을 실제 진단에 적용하기 위해서는 17번 염색체 상에 존재하는 유전자 중 가장 수적 안정성을 보이는 새로운 유전자를 발굴해낼 필요가 있다.

주요어 : 유방암, HER2, 17 번 염색체 동원체, 17 번 염색체의 다염색체성, 형광제자리보법, 참고 유전자

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