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Retrospective analysis of the association between human epidermal growth factor receptor 2 amplification and chromosome enumeration probe 17 status in patients with breast cancer

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Abstract. The aim of the present study was to identify potential human epidermal growth factor receptor 2 (*HER2*) amplification, according to American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) 2013 *HER2* testing guidelines, in patients previously determined not to possess *HER2* amplification, in accordance with previous 2007 guidelines. Potential discrepancies may arise from chromosome enumeration probe 17 (CEP17) amplification, deletion, polysomyor monosomy. *HER2*, CEP17, tumor protein p53 (*TP53*) and retinoic acid receptor α (*RARA*) genes from 67 patient specimens with suspected amplification, polysomy or monosomy of CEP17 were analyzed using fluorescence *in situ* hybridization. *HER2* status was interpreted using 2007 and 2013 ASCO *HER2* test guidelines as well as the reference genes *TP53* and *RARA*. According to ASCO/CAP2007 *HER2* guidelines, 20 patients exhibited *HER2* amplification (29.85%), 41 were without *HER2* amplification (including 25 with polysomy, 15 with monosomy and 1 with suspected monosomy plus co-amplification of *HER2* and CEP17) and the remaining 6 patients were equivocal. Using ASCO/CAP 2013 *HER2* guidelines, 49 patients exhibited *HER2* gene amplification (73.1%). The 29-patient increase included 6 originally at equivocal levels but now demonstrating amplification, 22 originally with polysomy but now revealing co-amplification, and 1 with suspected monosomy plus co-amplification of *HER2* and CEP17. According to *TP53* and *RARA*, *HER2* was amplified in 43 patients (64.1%). Using the revised guidelines, *HER2*, originally identified as amplified in 6 patients, was not amplified following the introduction of *TP53* and *RARA* control genes. Among these 6, 4 possessed normal *TP53* and *RARA*. The incidence of co-amplification of *HER2* and *CEP17* was 1.4% (21/1,518). *RARA* and *TP53* are suitable control genes to evaluate *HER2* status.

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Introduction

The human epidermal growth factor receptor 2 gene (*HER2*) is located on chromosome 17q12. In 1987, Slamon *et al* (1) proposed that the amplification of *HER2* was associated with breast cancer prognosis. Subsequently, *HER2* has been revealed to be amplified, or HER2 protein overexpressed, in between 20 and 30% of patients with breast cancer. These patients are generally diagnosed with high-grade cancer with increased rates of cell proliferation and a tendency to metastasize to the lymph nodes. Prognosis of these patients is markedly poorer compared with patients with breast cancer who do not overexpress HER2 (2-4). Herceptin/trastuzumab combined with chemotherapy may improve the quality of life of patients with HER2-positive breast cancer and prolong their disease-free survival time. Although a limited number have been described, occasional side effects of Herceptin treatment do occur, including cardiac toxicity that may weaken cardiac contractility, leading to cardiac insufficiency (5-9). On this basis, *HER2* status is an important marker for selecting suitable therapy.

The *HER2* test guidelines set out by the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) were updated in 2013 from the previous 2007 version; the evaluation standards of immunohistochemistry (IHC) and *in situ* hybridization (ISH) test results were revised in these guidelines (10,11). In China, HER2 IHC is extensively applied as a preliminary screen, whereas ISH is primarily considered a confirmatory test for *HER2* gene amplification, with the most common ISH test involving double-probe fluorescence (FISH). Distinctions between the 2013 and 2007 ASCO/CAP evaluation standards of double-probe FISH results are as follows: i) The threshold value of *HER2* amplification was adjusted to be ≥2.0 (≥2.2 in the 2007 version); ii) in the 2013 version, *HER2* amplification

was also defined as *HER2*/CEP17 <2.0 with mean HER2 copies/nucleus ≥6.0, or *HER2*/CEP17 ≥2.0 with mean *HER2* copies/nucleus <4.0. In the 2007 version, these values were considered to represent non-amplification (*HER2*/CEP17 <1.8) for patients identified with simultaneous *HER2* and chromosome 17 centromere locus amplification. However, in the 2013 version *HER2* is considered to be amplified in these patients and, therefore, these patients should be considered for *HER2*-targeted therapy. The aim of the present study was to evaluate the patients that did not exhibit *HER2* amplification by 2007 standards, but with potential *HER2* amplification by 2013 guidelines.

The selection of control genes for investigations using double probes is important. A control gene was selected for chromosome 17 to exclude influences of chromosome 17 polysomy in cancer cells. A second control gene was selected that is sufficiently distant from *HER2* so as to remain stable when *HER2* is amplified. On the basis of double-probe FISH studies by Troxell *et al* (12) and Varga *et al* (13), chromosome enumeration probe 17 (CEP17), tumor protein p53 (*TP53*) and retinoic acid receptor (*RARA*) were selected as controls for *HER2*.

In the present study, a retrospective analysis was performed to review *HER2* FISH-analyzed cases and to compare the 2007 and 2013 ASCO/CAP guidelines. Alterations in *HER2* status following the introduction of novel control genes were also determined. In addition, the effect of amplification or deletion, or polysomy of CEP17 in screening patients for targeted therapy was investigated.

Patients and methods

Samples. Specimens from 1518 patients with breast cancer were previously analyzed by *HER2* FISH between February 2011 and January 2015; samples were collected from 15 hospitals, including The First Affiliated Hospital of Chongqing Medical University, The Second Affiliated Hospital of Chongqing Medical University, Yongchuan Hospital Chongqing Medical University, The Hospital of Traditional Chinese Medicine of Chongqing, The Fifth People's Hospital of Chongqing, The Ninth People's Hospital of Chongqing, The People's Hospital of Chongqing Rongchang, The Centre's Hospital of Chongqqing Jiangjin, The People' Hospital of Chongqing Bishan, The People's Hospital of Chongqing Changshou, The People's Hospital of Chongqing Hechuan, The People's Hospital of Chongqing Qijiang, The People's Hospital of Chongqing Tongliang, The Centre's Hospital of Chongqing Fuling. FISH was performed for patients exhibiting medium to strong HER2 IHC levels prior to Herceptin administration, according to the ASCO/CAP 2013 criteria (11). From this FISH analysis, 67 specimenswith suspected amplification, polysomy and monosomy of CEP17 were selected for inclusion in the present study. This retrospective study was approved by the Chongqing Medical University ethics committee.

FISH. Paraffin-embedded tissue samples (from the 67 selected patients) were fixed in 10% neutral buffered formalin at room temperature for between 24 and 48 h, and were sectioned at a thickness of 4 μm. Hematoxylin and eosin staining for 5-10 min at room temperature was performed to label infiltrating carcinomas, and observation with an Olympus BX41 microscope (magnification, x40). FISH for *HER2*, CEP17, *TP53* and *RARA* was performed on paraffin sections according to the manufacturer's instructions (each individual probe of *HER2*, CEP17, *TP53* and *RARA* and solid tumor FISH testing protocol were obtained from Beijing GP Medical Technologies, Ltd.; China Medical Technologies Inc., Beijing, China). Information about marker probes is presented in Table I. Fluorescence signal observation, photography and analysis were performed using an Olympus BX51 fluorescence microscope (magnification, x100) and FISH software (version 2.0; Beijing GP Medical Technologies, Ltd.; China Medical Technologies Inc.). *HER2* status was interpreted according to the 2007 and 2013 ASCO/CAP *HER2* test guidelines as well as the control genes, *TP53* and *RARA*.

Table I. Labeled probes on chromosome 17.

Gene	Color	Marker site
Human epidermal growth factor receptor 2	Red	17q11.2-q12
Chromosome enumeration probe 17	Green	17p11.1-q11.1
Tumor protein p53	Green	17p13.1
Retinoic acid receptor α	Red	17q21.1

Results

FISH for CEP17 and *HER2*, as well as *TP53* and *RARA* was performed on 67 samples. According to ASCO/CAP 2007 guidelines, 20 patients exhibited *HER2* amplification (29.85%; 16 with CEP17 monosomy and 4 with partial CEP17 deletion), which was consistent with *HER2*/CEP17 ≥2.0 (Table II). On this basis, *HER2* was concluded to be amplified. A total of 6 patients were revealed to be equivocal for *HER2*/CEP17 (4 patients with 2.2> *HER2*/CEP17 >2.0 and 2 patients with 1.8 <*HER2*/CEP17 <2.0). A total of 41 patients did not experience *HER2* amplification, including 25 with polysomy (6 with CEP17 and *HER2* cluster-amplification and 19 with CEP17 and *HER2* punctiform-amplification), 15 with monosomy and 1 with suspected monosomy plus co-amplification of *HER2* and CEP17.

Table II presents *HER2* status according to various interpretation standards (ASCO/CAP 2007, ASCO/CAP 2013 and reference genes *TP53* or *RARA*). According to ASCO/CAP 2013 guidelines, 49 patients were diagnosed with *HER2* amplification (73%). The additional 29 patients who were not diagnosed with *HER2* amplification according to the 2007 criteria included 6 patients originally at the equivocal level but now demonstrating amplification (4 patients with *HER2*/CEP17 ≥2.0 and 2 patients with 1.8 < *HER2*/CEP17 <2.0 but *HER2* ≥6 signals/nucleus), 22 patients originally with polysomy but now exhibiting amplification (*HER2*/CEP17 <2, but *HER2* ≥6 signals/nucleus) and 1 patient with suspected monosomy plus co-amplification of *HER2* and CEP17 (*HER2*/CEP17 <2, but *HER2* ≥6 signals/nucleus).

Table II. Human epidermal growth factor 2 gene status according to distinct interpretation standards.

		ASCO/CAP 2013			Tumor protein p53 or retinoic acid receptor α		
ASCO/CAP 2007	n	Non-amplified	Equivocal	Amplified	Non-amplified	Equivocal	Amplified
Amplified	20	0	0	20	4	0	16
Equivocal	6	0	0	6	0	0	6
Non-amplified	41	18	0	23	20	0	21
Total	67	18	0	49	24	0	43

ASCO/CAP, American Society of Clinical Oncology/College of American Pathologists.

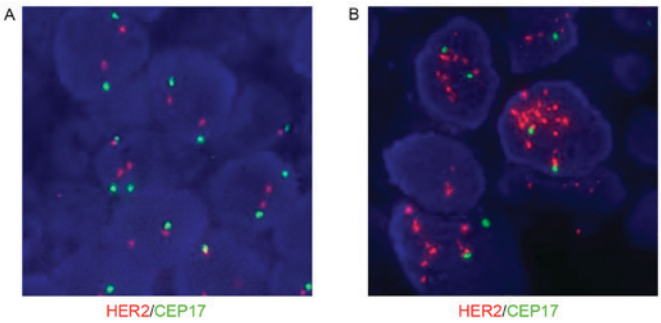

Figure 1. Common *HER2* (red) and CEP17 (green) status demonstrated using fluorescence *in situ* hybridization. Magnification, 100x10. (A) *HER2*/CEP17-negative group; no amplification of *HER2* or CEP17. (B) *HER2*/CEP17-positive group; amplification of *HER2* and normal CEP17. *HER2*, human epidermal growth factor receptor 2; CEP17, chromosome enumeration probe 17.

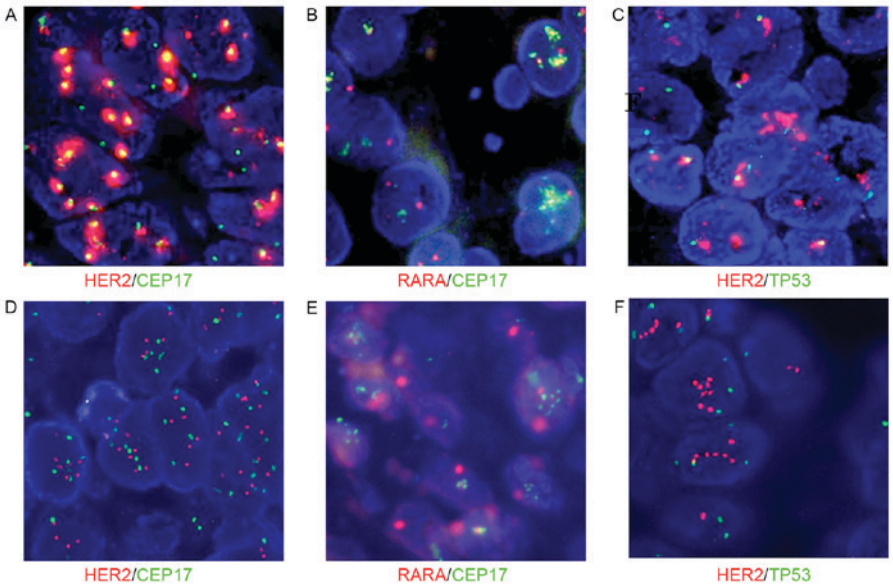

Figure 2. Co-amplification of *HER2* and CEP17 without polysomy, confirmed using fluorescence *in situ* hybridizationfor *TP53* and *RARA* genes. Magnification, 100x10. (A) *HER2*/CEP17, co-amplification of *HER2* (red) and CEP17 (green). (B) *RARA*/CEP17, normal *RARA* (red) and amplification of CEP17 (green). (C) *HER2*/*TP53*, *HER2* (red) amplification and normal *TP53* (green). (A-C) Samples from the same case, which exhibits a high level of co-amplification of *HER2* and CEP17. (D) *HER2*/CEP17, moderate co-amplification of *HER2* (red) and CEP17 (green). (E) *RARA*/CEP17, CEP17 (green) amplification and normal *RARA* (red). (F) *HER2*/*TP53*, *HER2* (red) amplification and normal *TP53* (green). (D-F) Samples from the same case, which was characterized by moderate amplification of *HER2* and CEP17. *HER2*, human epidermal growth factor receptor 2; CEP17, chromosome enumeration probe 17; *TP53*, tumor protein p53; *RARA*, retinoic acid receptor α.

The introduction of *TP53*, *RARA* and CEP17 as control genes indicated that *HER2* was amplified in 43 patients (64.2%). A total of 6 patients with *HER2* amplification according to ASCO/CAP 2013 guidelines did not exhibit amplification following the introduction of *TP53* and *RARA* control genes. Among these 6 patients, 4 exhibited normal *TP53* and *RARA*, partial CEP17 deletion, *HER2*/CEP17≥2, but *HER2*/*TP53* <2, *HER2*/*RARA* <2 and *HER2* <4 signals/nucleus, and the

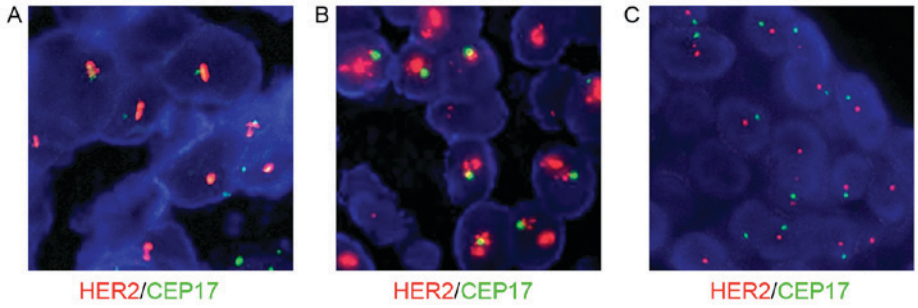

Figure 3. Fluorescence *in situ* hybridization analysis of chromosome 17 monosomy accompanied by mean irregular *HER2* (red) and CEP17 (green) status. Magnigication, 100x10. (A) *HER2*/CEP17, chromosome 17 monosomy accompany by co-amplification of *HER2* gene and CEP17. (B) *HER2*/CEP17, chromosome 17 monosomy accompany by amplification of *HER2*. (C) *HER2*/CEP17, monosomy not accompanied by amplification of *HER2* gene or CEP17. *HER2*, human epidermal growth factor receptor 2; CEP17, chromosome enumeration probe 17.

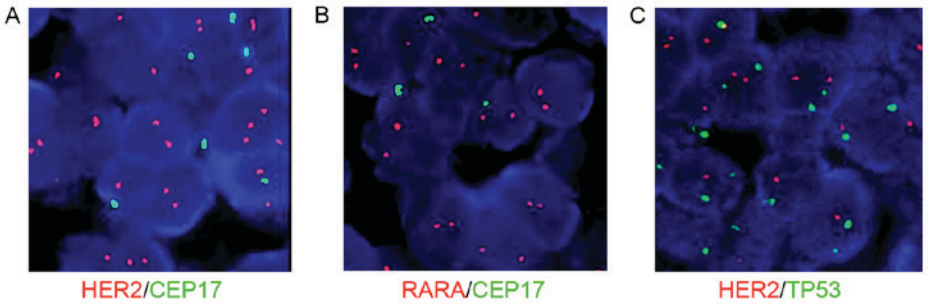

Figure 4. Fluorescence *in situ* hybridization analysis of normal *HER2* gene status and CEP17. Magnification, 100x10. (A) *HER2*/CEP17, partial CEP17 (green) deletion, normal *HER2* (red). (B) *RARA*/CEP17, normal *RARA* (red) gene and partial CEP17 (green) deletion. (C) *HER2*/*TP53*, normal *HER2* (red) and partial *TP53* (green) deletion. (A-C) Samples from the same case demonstrating CEP17 deletion. *HER2*, human epidermal growth factor receptor 2; CEP17, chromosome enumeration probe 17; *TP53*, tumor protein p53; *RARA*, retinoic acid receptor α.

remaining 2 patients demonstrated *HER2* ≥6 signals/nucleus and *HER2*/CEP17 <2, but *HER2*/*TP53* <2 and *HER2*/*RARA* <2, on which basis polysomy was defined. Of the 15 patients with monosomy, 3 patients exhibited normal *TP53* and *RARA*, therefore the number of monosomic patients was 12.

Using *TP53*, *RARA* and CEP17 as control genes, the incidence of chromosome 17 polysomy in 1,518 patients was 0.2% (3/1,518) and the incidence of monosomy was 0.8% (12/1,518). The incidence of co-amplification of *HER2* and *CEP12* was 1.4% (21/1518).

HER2 status was associated with the status of CEP17 and the reference genes. Fig. 1 demonstrates common *HER2* and CEP17 status using FISH. Fig. 2 reveals co-amplification of *HER2* and CEP17 polysomy. If only applying CEP17, *HER2*/CEP17 <2 and therefore *HER2* was not amplified according to the 2007 ASCO/CAP version, but was amplified according to the 2013 version (*HER2* ≥6 signals/nucleus). Fig. 3 reveals that chromosome 17 monosomy was accompanied by irregular *HER2* and CEP17 status. Fig. 4 demonstrates CEP17 deletion by FISH. If only applying CEP17, *HER2*/CEP17 ≥2 and therefore *HER2* was amplified according to the 2013 version of ASCO/CAP guidelines. However, FISH analysis of *TP53* and *RARA* revealed *HER2* to be normal.

Discussion

Samples without *HER2* amplification according to the ASCO/CAP 2007 *HER2* test guidelines may be classified as with *HER2* amplification according to the revised 2013 *HER2* test guidelines, particularly in contentious co-amplified specimens. This suggests that these patients may benefit from *HER2*-targeted medicine. Therefore, in the present study, FISH results from 1,518 patients were reviewed and 67 patients were identified with abnormal CEP17 signals, including suspicious co-amplification, depletion, polysomy and monosomy.

The incidence rate of co-amplification of *HER2* and CEP17 was 1.4% (21/1518), which demonstrates distinction from previous studies. Troxell *et al* (12) identified that 7/858 patients with cancer exhibited abnormal *HER2* and CEP17 (6 with breast cancer and 1 with ovarian carcinoma); the incidence rate of CEP17 amplification was 0.8%, whereas no *HER2* amplification was revealed in 3/7 patients. On this basis, the incidence rate of co-amplification was 0.47%. Varga *et al* (13) identified that 14 patients were diagnosed with co-amplification of >5,000 patients with breast cancer who underwent FISH analysis between 1999 and 2009, on the basis of which, the co-amplification incidence rate was 0.3%. Press (14) observed co-amplification in 2/2,600 patients with breast cancer, on the basis of which the co-amplification incidence rate was 0.08%. Gunn *et al* (15) selected 20 patients who exhibited unclear *HER2* status following routine FISH and IHC investigations, and identified *HER2* status through array-based comparative genomic hybridization (aCGH). Co-amplification of *HER2* and CEP17 was observed in 3/20 patients, for which the co-amplification rate was 15% in patients suspected to be positive for *HER2*; there was a tendency for a false negative result if based only on the *HER2*/CEP17 ratio. Marchio *et al* (16) randomly selected

18 patients (~8% of all cases) with a mean CEP17 >3 signals/nucleus to perform an aCGH test and identified that 17q containing the centromere locus was amplified in 11 patients, 17q excluding the centromere locus was amplified in 1 patient and was combined with true polysomy in 1 other patient, whereas amplification of only the centromere locus was identified in 5 patients. Therefore, the co-amplification incidence rate was 61.1% (11/18). On this basis, the overall co-amplification rate was 4.9%. Tse *et al* (17) selected 171 patients with a mean CEP17 signals/nucleus of >2.6 to analyze *HER2* FISH results from 5,683 patients. Novel control genes were introduced into the interpretation standards, *RARA* and *TP53*. Following the introduction of these control genes, *HER2* of 58 patients (43.9%) was defined to be amplified in 132 patients previously identified as non-amplified (on the 2007 ASCO/CAP criteria of *HER2*/CEP17). *HER2* gene amplification was identified in 13/14 patients at the threshold value. The ratio of *HER2*/CEP17 was at the threshold value of 1.8-2.2 or *HER2* gene copy 4.0-6.0. Additionally, *HER2* status continued to be defined as amplified in 25 patients in whom amplification was classified previously. The results observed a limited number of patients with polysomy, and the co-amplification rate was 1.8% [(58+13+25)/5863]. Egervari *et al* (18) investigated chromosome 17 polysomy and observed, using FISH, that 5/405 patients with breast cancer presented CEP17 ≥3 alongside *HER2* amplification, on the basis of which the co-amplification incidence was 1.23%. At the same time, Egervari *et al* (18) proposed that a pseudomorph of chromosome 17 polysomy was induced by CEP17 centromere locus amplification and therefore the incidence of chromosome 17 polysomy may be less.

Distinctions were observed in the incidence rates of co-amplification between the results of the present study and the aforementioned previous studies. A total of 22/1518 patients, analyzed using FISH in the present study, were observed to exhibit co-amplification, all of whom presented with medium to strong levels of HER2 IHC and excluded HER2 negative and weak specimens. If counting these negative or weak specimens, the incidence rate of co-amplification was ~0.55% (22/4016).

Currently, the definitions of polysomy and monosomy are as follows, polysomy occurs when an entire chromosome is duplicated one or more times, whereas monosomy is the result of complete deletion of a chromosome (11). With the inclusion of the control genes *TP53* and *RARA* in the present study, the incidence rate of polysomy was ~0.2% (3/1518), suggesting that true polysomy was less common than what was previously observed in the literature. In cases where increased levels of polysomy are detected, it may have occurred due to CEP17 amplification, as suggested by Zeng *et al* (19), whereas decreases in polysomy incidence rate may be caused by the section thickness being less than the diameter of cells (20,21). Chromosome 17 polysomy may indicate poor efficacy of cytotoxic medicines, leading to tumor metastasis (22,23), on the basis of which Herceptin and/or anthracyclines may be more suitable. However, whether patients with breast cancer who exhibit chromosome 17 polysomy should receive Herceptin therapy is disputed. Moelans *et al* (24) recommended not using the term 'polysomy 17' when in actuality a 'CEP17 copy number increase' was meant. Hanna *et al* (25) suggested that mean *HER2* copies/cell should replace the *HER2*/CEP17 ratio to evaluate *HER2* status.

Currently, compared with polysomy, investigations into monosomy are rare. Following the inclusion of *TP53* and *RARA* control genes in the present study, the number of patients with monosomy was decreased from 15 to 12. The 3 discrepant cases experienced CEP17 deletion rather than true monosomy, leading to *HER2* false positives (*HER2*/CEP17 ≥2). Those patients with *HER2* amplification induced by true monosomy were not sensitive to targeted therapy and prognosis was unsatisfactory (26).

In the present study, no *TP53* or *RARA* amplification was identified in breast cancer cells. Therefore, *TP53* and *RARA* may be considered as control genes of *HER2*, suitable for the diagnosis of suspected *HER2* and CEP17 co-amplification. However, *TP53* and *RARA* only represent part of, not the whole of, chromosome 17.

Previous studies indicate that gene sequencing may be carried out directly on chromosome 17 based on aCGH (16). Observation using aCGH of whether *HER2* was amplified was the optimal method to evaluate gene status, which was expensive. It was reported that when chromosome 17 was in a complex gene status, whole gene tests were recommended as positive FISH results were consistent with results of aCGH tests (16).

In conclusion, *HER2* was previously determined to not be amplified in 29 patients but was revealed, through retrospective analysis in the present study, to be amplified according to ASCO/CAP 2013 *HER2* test guidelines. *HER2* in 23 patients which had previously been judged to not be amplified, was revealed to be amplified following the inclusion of *RARA* and *TP53* control genes. The distinction of *HER2* status is important as it enables patients to receive targeted medicine. ASCO/CAP 2013 *HER2* test guidelines are more accurate than 2007 guidelines. In addition, *RARA* and *TP53* may be considered suitable control genes to evaluate *HER2* status.

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