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PRECLINICAL STUDY

Co-amplification of the *HER2* gene and chromosome 17 centromere: a potential diagnostic pitfall in HER2 testing in breast cancer

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Abstract Co-amplification of the centromere on chromosome 17 (*CEP17*) and *HER2* can occur in breast cancer. Such aberrant patterns (clusters) on *CEP17* can be misleading to calculate the *HER2/CEP17* ratio, and thus underreporting of *HER2* amplification. We identified 14 breast cancers retrospectively with *HER2/CEP17* co-amplification and performed FISH (fluorescence in situ hybridization) with additional chromosome 17 probes (17p11.1–q11.1, 17p11.2–p12, TP*53* on 17p13.1, *RARA* on 17q21.1–3 and *TOP2* on 17q21.3–22) to characterize the spanning of the amplicon in these cases. Furthermore, the *HER2* status was analyzed by means of *HER2* silver in situ hybridization (SISH) and immunohistochemistry (IHC). The co-amplification of *HER2/CEP17* was compared between the three institutions. *TP53* was eusomic in all cases, 17p11.2–p12 in 79% (11/14), whereas 17p11.1–q11.1 showed chromosomal gain in all cases. *RARA* was amplified in 10/14 cases (71%) and TOP2 in 3/14 cases (21%). *HER2* was amplified with FISH/SISH in all 14 cases. 9/14 tumors were 3+ IHC positive (64%) and 3 cases were 2+ IHC positive. In our cohort the *CEP17* amplicon almost always involves the *HER2* but not the *TOP2* locus. Overall agreement on *HER2/CEP17* ratio (when applying ASCO/CAP guidelines) was only 64% (9/14 cases) between the institutions. Discrepant ratios varied from 1.1 to 14.3. The *HER2/CEP17* co-amplification is not defined in the ASCO/CAP guidelines, and may result in inaccurate *HER2*-FISH/SISH status, particularly if only the calculated *HER2/CEP17* ratio is reported. It is recommended to report separate *CEP17* and *HER2* signals in complex *HER2/CEP17* patterns.

Keywords HER2 · CEP17 · Co-amplification · FISH · Breast cancer

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Introduction

Therapeutic response to Herceptin™ in HER2 positive breast cancer can be predicted by the HER2 status in routine diagnostic testing, which has been established by IHC and in situ hybridization (FISH/SISH/CISH) technology [30]. Depending on the applied test, the current diagnostic ASCO/CAP guidelines require different signal values for the evaluation of the *HER2* gene status [30]. When using FISH with the CEP17 control, a *HER2/CEP17* ratio >2.2 is necessary. If FISH, SISH or CISH (chromogenic ISH) is used without a CEP17 control, more than 6 gene copies or clusters of the *HER2* gene are sufficient to determine the HER2 status as positive [6, 7, 30]. If we deal with aberrant patterns, such as clustering of *CEP17* and/or *HER2*, standard ASCO/CAP criteria for FISH testing can be quite difficult to apply, as exact numeration of *CEP17* and *HER2* copy signals becomes difficult. Precise algorithm for the interpretation of double clustering is not defined in the ASCO/CAP guidelines. As in co-amplified cases, the *HER2/CEP17* ratio per se will be both mathematically and biologically useless if the *HER2* signal count becomes nearly the same as the *CEP17* signal count.

Co-amplification of both the *HER2* and *CEP17* region is a rare event, occurring in less than 1% of the breast cancer cases tested routinely [17, 23].

In this retrospective study, we present a comprehensive analysis of 14 breast cancer cases with cluster forming chromosomal gains on both *CEP17* and the *HER2* gene by FISH testing. We compared the evaluation of these cases and the standard calculation of the *HER2/CEP17* ratio among the three participating institutes. Large *HER2* amplicons can overlap numerous genes on 17q and 17p. Additionally, we tested the potential amplification status of adjacent genes on 17q and 17p, including *TOP2* (Topoisomerase 2A), *RARA* (Retinoic Acid Receptor Alpha) and *TP53*. *CEP17* was examined using two different probes covering centromeric and pericentromeric gene sequences of different lengths. Finally, we correlated the amplification status and ratios in all cases to the IHC results of HER2.

Materials and methods

Study cohort

Fourteen breast cancer cases with an amplified centromeric region of the chromosome 17 from routine HER2-FISH testing were retrieved from the diagnostic archives at the Institute of Surgical Pathology, University Hospital Zurich, Switzerland and from the Institute of Pathology, Cantonal Hospital St. Gallen, Switzerland. The age of the patients ranged between 39 and 76 years (mean age 61.1 years). All but one tumor corresponded histologically to invasive ductal carcinoma and one case was an intracystic papillary carcinoma with invasive components (Table 1). Seven cases were pT1c, four cases pT2, one case pT3, and two cases pT4. Axillary lymph node metastases were present in 11 patients and the lymph node status was not known for one patient (Nr. 14). Eleven carcinomas were hormone receptor positive. The study was approved by the project review board at the Institute of Surgical Pathology, University Hospital Zurich, Switzerland.

For the study, paraffin blocks of surgical specimens were used in 12 cases and core biopsies in 2 cases.

Methods

Fluorescence in situ hybridization (FISH)

Paraffin embedded sections with a thickness of two micrometers were used for all fluorescence in situ hybridization analyses. All procedures for the FISH analyses were carried out by following the recommended protocol of the manufacturers. Probe mixes were hybridized at 37°C between 14 and 20 h, washed in Rapid-Wash-Solution I at 73°C for 5 min, Rapid-Wash-Solution II and H_2O for 7 min, air dried and counterstained with DAPI. The reactions were evaluated using an Olympus computer guided fluorescence microscope (BX61, Olympus Schweiz AG, Volketswil, Switzerland). Each case was accompanied by a corresponding hematoxylin and eosin (H&E) stain in order to identify the invasive tumor component. The *HER2* status was analyzed in all 14 cases by the participating institutes (Zurich and Cleveland). Seven of the 14 cases from St.Gallen were tested during the weekly routine FISH

Table 1 Clinico-pathological parameters of the patients

Case Nr.	Age (years)	Histology	TNM stage	ER	PR
1	63	Invasive ductal carcinoma	pT2, pN1	neg	neg
2	71	Invasive ductal carcinoma	pT3, pN1	5%	5%
3	73	Invasive ductal carcinoma	pT4b, pN1	60%	neg
4	76	Invasive ductal carcinoma	pT2, pN1	1%	1%
5	61	Invasive ductal carcinoma	pT1c (m), pN1	90%	80%
6	59	Invasive ductal carcinoma	pT1c (m), pN1	neg	100%
7	53	Invasive ductal carcinoma	pT4b, pN2	30%	20%
8	64	Invasive ductal carcinoma	pT2, pN1	40%	neg
9	39	Invasive ductal carcinoma	pT1c, pN1	100%	90%
10	60	Invasive papillary carcinoma	pT1c, pN0	4%	neg
11	49	Invasive ductal carcinoma	pT1c, pN2	14%	3%
12	60	Invasive ductal carcinoma	pT1c, pN0	80%	neg
13	74	Invasive ductal carcinoma	pT2, pN1	75%	neg
14	54	Invasive ductal carcinoma	pT1c, pNx	85%	95%

ER estrogen receptors, *PR* progesterone receptors. Positivity for hormone receptors is indicated as percentage of positively stained invasive tumor cells

diagnostics. All other tests were carried out at the Institute of Surgical Pathology, University Hospital Zurich, Switzerland.

HER2 gene

The *HER2* gene was tested by using a dual fluorescence kit (PathVysion™, Vysis, Abbott AG, Diagnostic Division Baar, Switzerland) containing the *HER2* gene (17q11.2–q12, directly labeled with fluorescent spectrum orange) and *CEP17* (17p11.1–q11.1, directly labeled with fluorescent spectrum green) (Fig. 2).

CEP17–D17Z1 (centromeric region of chromosome 17) locus 1

For this locus, a kit labeling the region 17p11.1–q11.1 (D17Z1) of *CEP17* (Vysis, Abbott AG, Diagnostic Division Baar, Switzerland) was used (Fig. 2). The probe contained a direct fluorescent labeled area with spectrum aqua.

CEP17–D17S122/HER2 (centromeric region/HER2 gene on chromosome 17) locus 2

For this locus, the probe *D17S122*, covering the region 17p11.2–p12, was used (Fig 2). The area was visualized by direct fluorescent labeling containing spectrum green. The reactions were carried out on all 14 cases at the Section of Molecular Pathology, Cleveland Clinic. All probes for this locus (along with *HER2*) were graciously provided by Dr. Robert Jenkins of the Mayo Clinic (Rochester, Minnesota, USA). Spectrum green labeled *D17S122-1* (RP11-465O5), *D17S122-2* (RP11-726O12), *D17S122-3* (RP11-924A14) and *D17S122-4* (RP11-136M15). Spectrum orange labeled *HER2 BAC1* (RP11-94L15), and *HER2 BAC2* (CTD-2019C10). All these probes were used to generate the *HER2/D17S122* probe cocktail. HER2 was labeled with spectrum orange and *D17S122* was labeled with spectrum green. The slides were probed with 1 µl *HER2* probe, 2 µl *D17S122* probe, 2 µl human placenta DNA and 5 µl hybrisol and incubated overnight at 37°C.

RARA gene

For the *RARA* locus, a combined probe of *LSI®PML/RARA* (Vysis, Abbott AG, Diagnostic Division Baar, Switzerland) was applied. The *RARA* gene (17q21.1–q21.3) was directly labeled with the fluorescent spectrum green probe, the *LSI®PML* (15q22, not assessed in the study) was directly labeled with fluorescent spectrum orange (Fig. 2).

TOP2 gene

For the *TOP2* gene, a triple probe (Vysis, Abbott AG, Diagnostic Division Baar, Switzerland) was applied containing the *HER2* gene (17q11.2–q12, labeled with spectrum green), the centromere *CEP17* (17p11.1–q11.1, labeled with spectrum aqua) and the *TOP2* gene (17q21.3–q22, labeled with spectrum orange) (Fig. 2).

TP53 gene

A dual probe (Vysis, Abbott AG, Diagnostic Division Baar, Switzerland) was used for the *TP53* gene, containing the *TP53* gene (17p13.1, labeled with fluorescent spectrum orange) and *CEP17* (17p11.1–q11.1, labeled with fluorescent spectrum green) (Fig. 2).

Silver-enhanced in situ hybridization (SISH) for HER2 and CEP17

In seven cases (Nr. 1–7) from the Zurich cohort, the HER2 status was also investigated with a silver enhanced in situ hybridization dual probe (Inform, Ventana Medical Systems, Tucson, AZ, USA).

The *HER2* DNA probe (catalog Nr.: 780-4332) was directly labeled with silver and the chromosome 17 probe (catalog Nr.: 780-4331) was labeled with red. The signals were detected with the ultraView SISH detection kit and the ultraView red ISH detection kit. The whole process was completely automated using Ventana's Benchmark autostainers according to the manufacturer's protocol. A corresponding H&E control slide was available in each case for the SISH analysis.

Immunohistochemistry for HER2

Paraffin-embedded sections with a thickness of 2 µm were used for the immunohistochemistry. Detection of the HER2 protein was performed with the Ventana Benchmark automated staining system using Ventana reagents (Ventana Medical Systems, Basel, Switzerland) for the entire procedure. Primary antibodies were detected using the iVIEW DAB detection kit and the signal was enhanced using the amplification kit. The following marker was used: Pathway anti-HER2, 4B5 (Ventana, Basel, Switzerland; ready to use without further dilution; concentration, 6 µg/ml).

Guidelines used to interpret the in situ hybridization (FISH and SISH) in CEP17 and HER2

The ASCO/CAP guidelines were used to interpret the signals in the FISH and SISH analyses [12, 30]. The

number of signal copies for *CEP17* and *HER2* was calculated for each probe. Optical not separable clusters were set to 16 copies in the FISH analyses. Furthermore, the ratios of the dual probes were evaluated. SISH small clusters were set to 6 copies and larger clusters to 12 copies. Similarly, a ratio >2.2 was set as an amplified status and ratios <1.8 were negative. We used definitions from recently published recommendations by Vance et al. when we were dealing with intratumoral heterogeneity: at least 2 (and up to 4) representative fields from the invasive areas were evaluated. If more than 50% of the invasive tumor cells in these areas had a *HER2/CEP17* ratio higher than 2.2, we considered the tumor as amplified. We used these criteria to examine all the gene regions named above: *HER2*, *CEP17*, *RARA*, *TOP2*, and *TP53* [16, 25, 27].

The counting and interpretation of the FISH-*HER2* signals were performed individually at each institute (ZV, GB, RT, ZW, YS, DK, CO). Signals for SISH-*HER2* and FISH-*TOP2* were analyzed and counted in Zurich (ZV). The reading and counting of FISH-*CEP17* (1), FISH-*TP53* and FISH-*RARA* signals were carried out both in Zurich (ZV) and in St.Gallen (DK, CO). Finally, the evaluation of the FISH-*D17S122/HER2* signals was performed in Cleveland (RT, ZW, YS).

Guidelines for the interpretation of the HER2 immunohistochemistry

The ASCO/CAP guidelines were used to interpret the staining of the HER2 protein expression and scored as follows: 0 (no staining), 1+ (weak and incomplete membrane staining), 2+ (strong, complete membrane staining in less than 30% of the invasive tumor cells or weak/moderate heterogeneous complete staining in more than 10% of the invasive tumor cells), and 3+ (strong complete homogenous membrane staining in more than 30% of the invasive tumor cells) [12, 30].

Interpretation of HER2 Status by HER2/CEP17 ratios

Although both the *HER2* gene and the *CEP17* region exhibited 'amplification' separately in each case, there was a huge discrepancy regarding the exact *HER2* status of these tumors (Table 3). In 5 of 14 cases (35%) the ratios ranged from 1.1 to 14.3 between the three institutions. These problematic cases included 2 tumors with an immunoreactivity of 3+ and 3 tumors with an immunoreactivity of 2+. In 9 of 14 cases (64%) the institutions reached an agreement on the *HER2* status as amplified or non-amplified, even though the individual ratios varied. We used the criteria mentioned above in the guidelines for the in situ hybridization for all the gene regions [16, 25, 27]. In one institute, the exact method of counting required an electronic excel data sheet as described in previous publications [16, 25, 27]. The other two institutions used direct counting on the computer screen and/or on the fluorescence microscope. Discrepant signal interpretation was principally due to the choice of either reporting the *HER2/CEP17* ratio or to reporting the raw signal data. For example, counting 60 cells (as happened in case Nr. 8) showed 1037 *HER2* and 1007 *CEP17* signals. The *HER2* gene count of 17.28 would imply amplification, whereas the *CEP17* gene count of 16.78 would mean high chromosomal gain using the recommendations suggested by Viale et al. in his discussion for real polysomic cases [16, 25, 27]. According to the ASCO/CAP guidelines on the other hand, this case is classified as non-amplified as the *HER2/CEP17* ratio is 'only' 1.03 [12, 30].

Results

In situ hybridization

HER2-FISH

In six cases (Nrs. 4,8,10,11,12,14) we found multiple large clusters, in four cases (Nrs. 1, 2, 5, 7) clusters and gene copies (>5), in four cases (Nrs. 3, 6, 9, 13) multiple copies of the *HER2* gene were present (>5, up to 20–25 gene copies) (Figs. 1, 3a, 4a, b; Tables 2, 3).

HER2-CEP17 SISH

Seven cases (case Nrs. 1–7) were tested with SISH. In all 7 cases, the *HER2* gene was present either in >5 copies and/or in large clusters (100%) (Fig. 3c). In 3 of 7 cases, both *HER2* and *CEP17* were present in clusters (Nrs. 1, 2, 4). In cases Nr. 5 and Nr. 7, *CEP17* and the *HER2* gene showed large cluster formations as well as multiple gene copies (up to 8 gene copies). In case Nr. 6 both *CEP17* and *HER2* displayed multiple gene copies (*CEP17* up to 8 copies and the *HER2* gene up to 25 copies). Case Nr. 3 showed >5 HER2 gene copies (5–8) and up to 8 CEP17 copies.

CEP17 (locus 1)

CEP17 (locus 1) was analyzed both with a dual *HER2* probe as well as with a single probe.

CEP17 (locus 1) dual probe:

All 14 cases (100%) revealed cluster formation and/or multiple copies of *CEP17* (locus 1) (Fig. 1, Table 2). In 6 cases (Nrs. 4, 5, 9, 10, 12, 13) *CEP17* was visualized as solitary large clusters (Fig. 3d, 4c). In another 6 cases (Nrs. 1, 2, 7, 8,

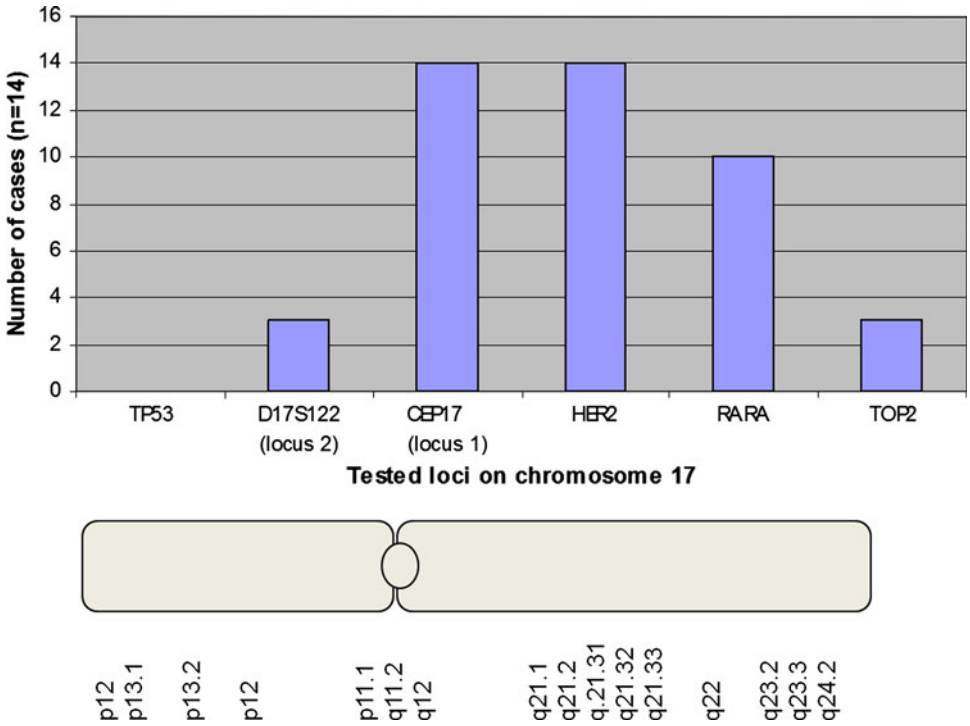

Fig. 1 Graphical representation of amplified gene regions on chromosome 17. Amplification is meant as absolute gene copy number of at least 6 or the presence of clusters in more than 50% of the tested invasive tumor cells

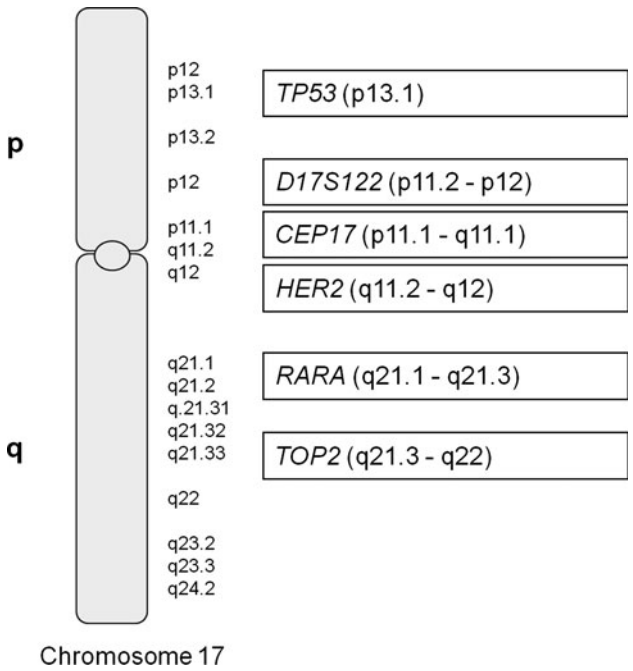

Fig. 2 Anatomical portrayal of the investigated gene loci on chromosome 17

11, 14), we found both clusters and up to 8 gene copies. Two cases (Nrs. 3, 6) revealed up to 8–12 gene copies.

CEP17 (locus 1) single probe

Case Nr. 12 could not be evaluated for this region with the single probe, as no clear signals could be achieved after repeated testing. Large clusters were seen in 3 cases (Nrs. 1, 2, 4). In 4 cases (Nrs. 5, 7, 8, 14), *CEP17* was found in clusters and up to 8 gene copies. Two cases (Nrs. 3, 6) revealed up to 8 gene copies and in case Nrs. 9, 10, 11, 13 there were small clusters.

D17S122/HER2 (locus 2)

Three of 14 cases (21%) showed multiple copies of *D17S122* (locus 2) (Table 2). The average number of copies was 5.5 (Nr. 3), 3.1 (Nr. 6) and 7.1 (Nr. 7). *HER2* was present with >5 copies in 9 of 14 cases and with <5 copies in 5 of 14 cases (Fig. 1).

RARA gene

Ten of 14 cases (71%) revealed amplification of the *RARA* gene (Table 2; Figs. 1, 3b). In 2 cases (Nrs. 9 and 10), amplification was a focal finding (at least in 60 cells) and in all the other cases, tumor cells were diffusely amplified. In 2 cases (Nrs. 1, 4), large clusters and up to 8 gene copies were detected. In 5 cases (Nrs. 7, 8, 9, 10, 14), *RARA* was present in large clusters, and in 3 cases (Nrs. 5, 6, 11) in multiple copies (up to 8). Case Nr. 12 could not be analyzed with this probe as no clear signals were visible.

TOP2 gene

Three of 14 cases (21%) showed an amplified *TOP2* region with up to 8 separate gene copies visible in two cases

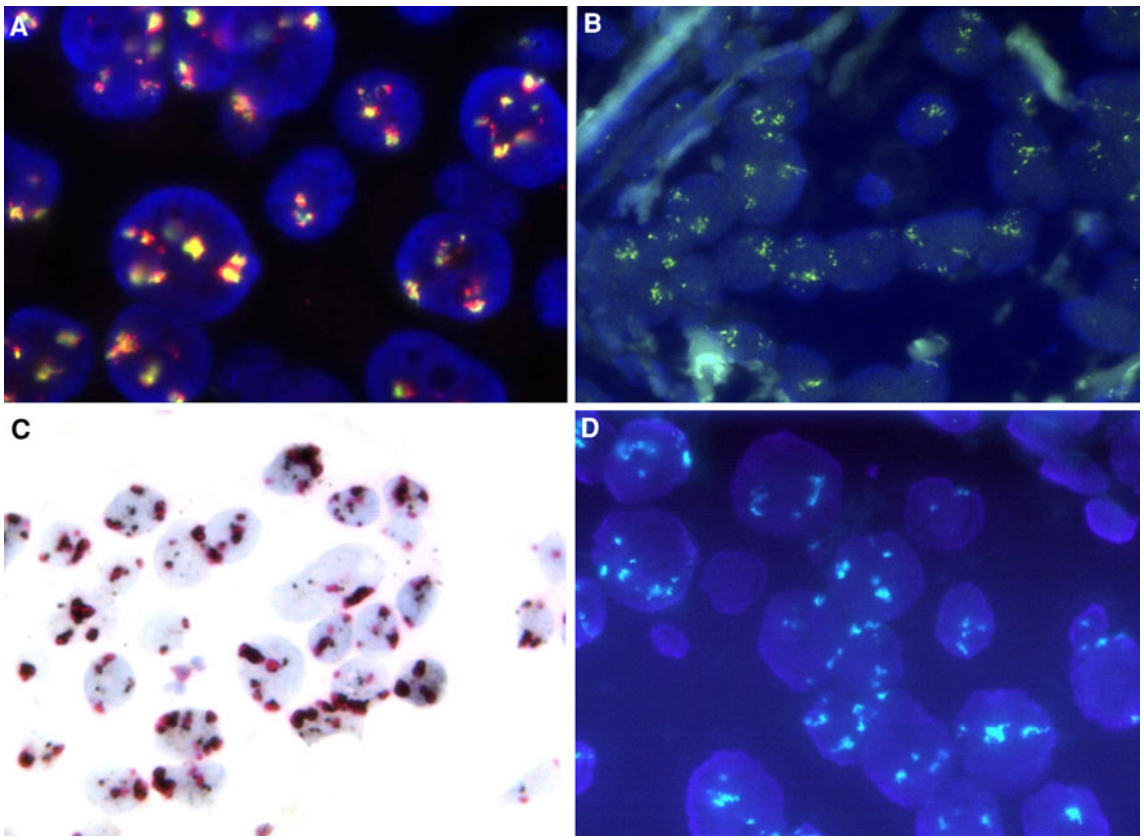

Fig. 3 Different amplification patterns in cluster forming chromosomal gain on 17q. **a** FISH *HER2/CEP17* dual probe (Vysis): *HER2*: *orange*, *CEP17*: *green*, both are in partially overlapping clusters. **b** FISH *RARA/LSI*® *PML* dual probe (Vysis): *RARA* clusters in *green coloration*, LSI®PML not photographed. **c** SISH *HER2/CEP17* dual probe (Ventana), *HER2*: *black*, *CEP17*: *red*, both genes display large overlapping clusters. **d** FISH *CEP17* single probe (Vysis): *CEP17*: in spectrum aqua, in large clusters

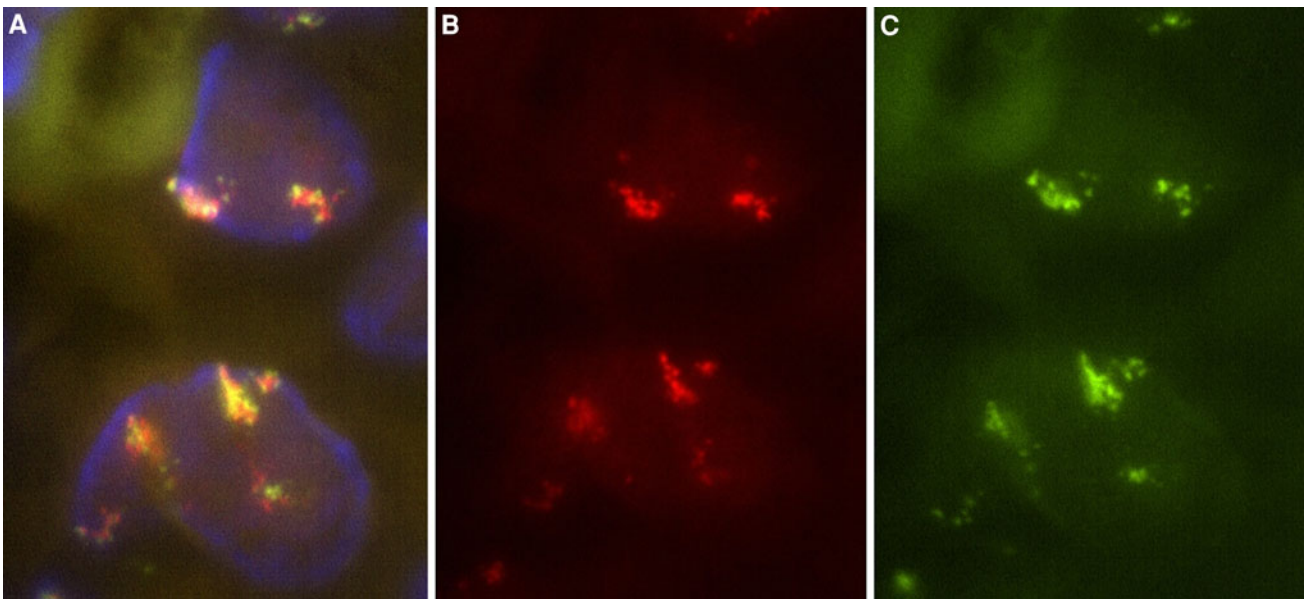

Fig. 4 High magnification of *HER2/CEP17* amplicons in the same two carcinoma cells: **a** FISH *HER2/CEP17* dual probe (Vysis): *HER2*: *orange*, *CEP17*: *green*, both signals in partially overlapping clusters. **b** FISH *HER2* (dual probe with *CEP17*) (Vysis): *HER2*: orange in large clusters, *CEP17* is switched out. **c** FISH *CEP17* (dual probe with *HER2*) (Vysis): *CEP17*: *green* in large clusters, *HER2* is not photographed

Table 2 Summary of the fluorescence in situ hybridization (FISH) on the tested loci on chromosome 17. Numbers indicate number of cases (total $n = 14$)

	Probe (locus)	Eusomic gene	Clusters and/ or gene copies >5	Intratumoral heterogeneity	No signals
TP53	17p13.1	14	0	0	0
D17S122 (locus 2)	17p11.2–p12	11	3	0	0
CEP17 (locus 1)	17p11.1–q11.1	0	14	3	0
HER2	17q11.2–q12	0	14	3	0
RARA	17q21.1–q21.3	3	10	2	1
TOP2	17q21.3–q22	11	3	3	0

CEP17 gene status refers to the whole cohort (including summarized results both with a dual as well as with the single probe)

Table 3 Differential calculation of *HER2/CEP17* ratios in the participating institutions. IHC: Immunohistochemistry. FISH: fluorescence in situ hybridization

Case Nr.	HER2 IHC	Ratio: *HER2/CEP17* FISH Institution I	Ratio: *HER2/CEP17* FISH Institution II	Ratio: *HER2/CEP17* FISH Institution III	Diagnostic concordance
1	3+	>2.2	7.6	Not done	Yes
2	3+	>2.2	10.7	Not done	Yes
3	0	1.0	0.4	Not done	Yes
4	3+	>2.2	17.3	Not done	Yes
5	3+	>2.2	5.4	Not done	Yes
6	3+	>2.2	6.3	Not done	Yes
7	3+	>2.2	2.8	Not done	Yes
8	3+	>2.2	14.3	1.1	No
9	2+	<1.8	2.5	0.55	No
10	3+	>2.2	1.0	1.28	No
11	3+	>2.2	13.4	2.89	Yes
12	2+	>2.2	1.3	1.16	No
13	1+	<1.8	1.2	0.96	Yes
14	2+	>2.2	8.4	1.9	No
Positive HER2 status	9/14 (64%)	11/14 (78%)	10/14 (71%)	1/7 (14%)	
Disconcordant cases					5/14 (35%)

(Nrs. 1, 14), and in case Nr. 8 large clusters were visible (Fig. 1; Table 2). The *CEP17* (locus 1) gene status, analyzed along with *TOP2*, was found to be identical to the *CEP17* gene status analyzed with the *HER2/CEP17* dual probe.

TP53 gene

We could not identify any cases with *TP53* amplification or chromosomal gain (Fig. 1; Table 2). All 14 cases revealed 2–3 gene copies of this gene (0/14), indicating that there are no cases with a high chromosomal gain in our cohort. As the dual probe also contained the *CEP17* region, this was also assessed. We found a 100% concordance when compared to the dual *HER2/CEP17* (locus 1) probe, pointing to an amplified *CEP17* (locus 1) in all cases.

HER2 immunohistochemistry

Nine of 14 cases (64%) displayed score 3 membranous stains (Table 3). Eight of these were also FISH positive (89%). One 3+ tumor (Nr. 10) displayed scattered areas (hotspots with at least 60 cells) containing amplified cells with FISH. Three of 14 cases were scored as 2+ (21.5%) and all these 2+ cases were problematic in the FISH analysis, as the ratio calculation (*HER2/CEP17*) resulted in diverging values. One case (Nr. 13) was scored as 1+ (7%). This case had a heterogenous pattern between the HER2-IHC and the *HER2*-FISH as only scattered areas (hotspots of at least 60 cells) were amplified with FISH. This case (Nr. 13) had up to 6 *HER2* gene copies and small *CEP17* clusters. If counting the absolute *HER2* gene copy number, 6 gene copies would qualify this case as amplified.

Calculating the *HER2/CEP17* ratio (ratios: 1.2, 1.8 and 0.96, respectively) (Table 3), this resulted in a non-amplified status. One case (Nr. 3) showed a negative immunostaining (7%). This case had a minimum of 8 *HER2* and 8 *CEP17* gene copies. The absolute *HER2* gene copy number would be sufficient for a positive HER2 status. On the other hand, calculating the *HER2/CEP17* ratio (0.4 and 1.0, respectively) results in a negative status.

Discussion

We identified *CEP17/HER2* co-amplification in a series of *HER2*-FISH assays, and as a result we investigated the nature of the chromosomal region spanning *HER2* and *CEP17* with different FISH probes to additional loci (*TP53*, *17p11.1–q11.1*, *17p11.2–p12*, *RARA*, and *TOP2*). We also examined the *HER2* gene with SISH and the HER2 protein expression with IHC. The assessment of the co-amplification by FISH was highly diverse in the different laboratories due to missing ASCO/CAP *HER2* assay guidelines for this situation. Twelve of 14 cases showing a *CEP17/HER2* co-amplification had a *HER2* score of either 3+ or 2+.

During the last decade the diagnosis of breast cancer has become standardized worldwide by identifying a positive HER2 status by means of immunohistochemistry and FISH [12, 20, 30]. *HER2*-FISH and HER2 immunohistochemistry assays represent predictive oncologic assays, whereby the staining intensity and gene alterations are characterized by distinct cut-off values of positive signals, and a clear algorithm for the interpretation of the signals exists [12, 20, 30]. Using *HER2* test kits approved by the US Food and Drug Administration (FDA), a strong circular membranous stain in more than 30% of the tested tumor area is defined as a positive HER2 status via immunohistochemistry [12, 30]. Although a positive *HER2* status by means of in situ hybridization has been distinctly defined according to the ASCO-CAP guidelines, there are subtle differences in classifying an amplified status when using different labeling technologies [20, 30].

The most widely used assay, the *HER2*-FISH analysis, requires a ratio >2.2 for the copy numbers of *HER2* to *CEP17* for an amplified status [20, 30]. The ASCO/CAP guidelines, however, do not define the role of a chromosomal gain [20, 30]. A *HER2/CEP17* ratio can be misleading in cases showing an extremely high chromosomal gain, as seen in many of our discrepant cases (Figs. 3, 4). On the other hand, the presence of large clusters of the *HER2* gene or more than six, respectively, 10 dots of the *HER2* gene are sufficient to deal with a positive HER2 status when using silver or chromogenic enhanced in situ hybridization technology (CISH, SISH) [30].

An accurate count of the copy number of the *CEP17* region turned out to be quite problematic in our 14 diagnostic *HER2*-FISH cases, as both the *CEP17* region and the *HER2* gene occurred as clusters, to a greater extent as large and to a lesser extent as small clusters. As the exact copy number of both *CEP17* and the *HER2* gene are required for the assessment of the *HER2/CEP17* ratio using ASCO/CAP criteria, we were confronted by a scenario for which there is no recommendation in the current guidelines [30].

By using SISH technology, however, 5 of the 7 tested cases would easily have qualified as amplified for *HER2*, as the presence of *HER2* clusters alone adequately fulfill these criteria [30]. Similarly, in a recent paper Marciò et al. [14] showed that >6 *HER2* gene copies is considered as a therapeutically important amplification, as true *CEP17* polysomy very rarely occurs. In this microarray-based comparative genomic hybridization (aCGH) study, Marciò et al. [14] demonstrated that an abnormal *CEP17* copy number is most likely due to the amplification of the CEP17 region regardless of the copy number gains of the short and long arms.

As none of our cases showed an amplification of the *TP53* gene, but instead a chromosomal gain with 2–3 signals, this indicates that we do not have true polysomic cases in this series. All our cases appear to show large amplicons of the *HER2* gene spanning at variable lengths to the centromeric region. Theoretically, it is possible that large *HER2* and *CEP17* signals are optically inseparable by using one single bandpass filter.

We compared the interpretation of the cluster formation on *CEP17* and the *HER2* gene by using the ASCO/CAP guidelines between the three participating institutions and found an enormous discrepancy in the final results. An agreement on the FISH-*HER2* status using the *HER2/CEP17* ratio as negative or positive could only be reached in 9 of 14 cases (64%) even though cluster formation of the *HER2* gene was present in most cases. The ratios varied between 1.1 and 14.3 in the discrepant cases. This was due to the simple mathematical fact that the *CEP17* and *HER2* copy numbers were equal or very similar resulting in a practically unusable ratio.

In such situations, following other recommendations, an absolute *HER2* gene copy count of >6 will fulfill the criteria for a *HER2* positive status in all cases (100%) [16, 25, 27]. Although not included in the ASCO/CAP guidelines, there are papers that propagate their 'own made' criteria, at least for the numeration of the *HER2* gene in the case of cluster formation. Tight clustering of the *HER2* gene was defined by Simon et al. [21] as being equivalent to 5 gene copies by FISH testing. The classification of small *HER2* clusters as being equal to 6 to 10 copies or of large clusters being equal to >10 copies in CISH *HER2* testing by Tanner et al. [22] seem somewhat

arbitrary but quite practical if it is about ratio calculation. Then again, several authors avoid defining a random copy number for the *HER2* clusters and instead call them 'classical clustering' or admit an 'imprecise signal numeration' as proposed by Lebeau and Sauter [11, 20]. In co-amplified cases with optically unseparable clusters we set the definition as 16 copies for FISH and as 12 copies (large clusters), respectively, 6 copies (small clusters) for SISH. With this definition, we provided real gene counts, which reflect the amplification status of the cells more realistically than the ratios alone. Luckily enough, most cases showing *HER2* gene clustering lack a simultaneous cluster formation of CEP17 enabling an easy diagnostic decision on the *HER2* status [11, 20].

According to our knowledge, there is only one study available from 2006 by Troxell et al. on 7 cases with a corresponding editorial from M. Press, addressing the co-amplification of *CEP17* and the *HER2* gene and the question of how to deal with *HER2* testing in such settings [17, 23]. Troxell et al. [23] proposed an extended FISH analysis on the neighboring *RARA* gene adjacent to the *HER2* gene and also adding a *HER2* immunohistochemistry to the test. Five of their 7 cases were 3+ positive on a protein level and also exhibited an amplification on the neighboring *RARA* gene [23].

We took a different approach to characterize the adjacent gene regions in the 14 amplified *CEP17* regions. On the one hand, we used a second probe (D17S122) for the *CEP17* region, labeling a much shorter DNA sequence (17p11.2–p12) than the one in the Vysis kit (17p11.1–q11.1). Cluster formation of *CEP17* was detected in 3 of 14 cases with this shorter probe. In one case (Nr. 3) multiple separate signals of *CEP17* were seen as well. We extended the adjacent gene regions and additionally tested for the *RARA* gene, the distally located *TOP2* gene on the long arm, and the *TP53* gene on the short arm of chromosome 17. No amplification was detected for the *TP53* gene in any of the 14 cases, which corroborates with literature data. *TP53* mutations but no amplifications have been found in sporadic *HER2* positive breast cancer [29]. In a recent study, *TP53* protein overexpression was only detected in unamplified *CEP17* polysomic breast cancer cases [10]. The high frequency of *RARA* co-amplification in our study (10 of 14 cases, 71%) is very similar to what has already been reported [14, 23]. Co-amplification of the *RARA/TOP2/HER2* chromosomal regions can occur in other malignancies as well, as was recently reported in a case of acute myeloid leukemia [2]. It may be true that the *TOP2* gene is virtually always co-amplified with the *HER2* gene in breast cancer, nevertheless, co-amplification frequencies varying between 30 and 100% has been published [9, 15, 18]. Therefore, the low *TOP2/HER2* co-amplification ratio (21%) in our study probably represents a case selection bias.

In our study, all cases showed a *HER2/CEP17* co-amplification. Amplification of *CEP17* without involvement of the *HER2* gene has been reported in the literature. Marchio et al. [14] analyzed 5 cases with an amplified *CEP17* region, however, only one of these cases exhibited >8 *HER2* copies, classifying this case as non-amplified by the *HER2/CEP17* ratio.

The presence of multiple gene amplifications on chromosome 17 is a complex process potentially involving a large *HER2* amplicon with further altered telomeric genes such as *TOP2*, *RARA*, *GRB7*, *STARD3* [8, 26]. It is very likely that the *HER2* gene amplification is the first event in the amplicon formation followed by additional chromosomal changes in the telomeric regions [8, 26]. Deletions and amplifications of other genes have been shown to bear a predictive value in the response to targeted therapy such as anthracycline in *HER2* positive breast cancer [1, 8, 9, 18, 26].

Comparative genomic hybridization studies have shown that the long arm of chromosome 17 is particularly prone to genomic changes. Copy number gains have been identified most frequently on 17q (57% prevalence) [5]. The formation of large amplicons and the activation of proto-oncogenes probably occur through classical amplification mechanisms such as double minute formations (extrachromosomal units) and homogenously stained regions (as a component of a chromosome) [4, 19].

Between 1999 and 2009, ~5,000 FISH-*HER2* analyses were performed in Zurich and St.Gallen. *HER2/CEP17* co-amplification was diagnosed in 14 of these cases. Therefore, co-amplification of *CEP17* and the *HER2* gene is a rare event in breast cancer, occurring in less than 1% of the tested cases.

Accurate interpretation of increased *CEP17* and *HER2* copy numbers (6 to 10 copies or clusters) is of enormous importance, as false positive or a negative HER2 status can occur if testing is not done with consequence as well as with the correct calculation of the *HER2/CEP17* ratio [3, 13, 17, 23, 24, 28]. If multiple complex genetic alterations are detected on chromosome 17 at routine *HER2* testing, then careful evaluation of the *HER2* amplicon along with the potentially co-amplified neighboring genes, and additional immunohistochemistry for HER2 is necessary [17, 23].

In summary, our data indicate that a complex FISH pattern with *HER2/CEP17* co-amplification requires confirmatory HER2 analysis by immunohistochemistry. It is recommended to report raw FISH data, including *CEP17* signals and *HER2* signals as well as the *HER2* gene count as the clinically most relevant FISH parameters. Calculation of the *HER2/CEP17* ratio can be misleading as such patterns can easily be categorized as 'chromosomal gain' which can result in a 'false negative' *HER2* status.

Moreover, the testing of chromosomal loci lying far away from the *HER2* region, such as TP53, is very helpful in defining or ruling out true polysomy.

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