HER2 *in situ* hybridization in breast cancer: clinical implications of polysomy 17 and genetic heterogeneity

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Trastuzumab-containing therapy is a standard of care for patients with HER2+ breast cancer. HER2 status is routinely assigned using *in situ* hybridization to assess HER2 gene amplification, but interpretation of *in situ* hybridization results may be challenging in tumors with chromosome 17 polysomy or intratumoral genetic heterogeneity. Apparent chromosome 17 polysomy, defined by increased chromosome enumeration probe 17 (CEP17) signal number, is a common genetic aberration in breast cancer and represents an alternative mechanism for increasing HER2 copy number. Some studies have linked elevated CEP17 count ('polysomy') with adverse clinicopathologic features and HER2 overexpression, although there are numerous discrepancies in the literature. There is evidence that elevated CEP17 ('polysomy') count might account for trastuzumab response in tumors with normal HER2:CEP17 ratios. Nonetheless, recent studies establish that apparent 'polysomy' (CEP17 increase) is usually related to focal pericentromeric gains rather than true polysomy. Assigning HER2 status may also be complex where multiple cell subclones with distinct HER2 amplification characteristics coexist within the same tumor. Such genetic heterogeneity affects up to 40% of breast cancers when assessed according to a College of American Pathologists guideline, although other definitions have been proposed. Recent data have associated heterogeneity with unfavorable clinicopathologic variables and poor prognosis. Genetically heterogeneous tumors harboring HER2-amplified subclones have the potential to benefit from trastuzumab, but this has yet to be evaluated in clinical studies. In this review, we discuss the implications of apparent polysomy 17 and genetic heterogeneity for assigning HER2 status in clinical practice. Among our recommendations, we support the use of mean HER2 copy number rather than HER2:CEP17 ratio to define HER2 positivity in cases where coamplification of the centromere might mask HER2 amplification. We also highlight a need to harmonize *in situ* hybridization scoring methodology to support accurate HER2 status determination, particularly where there is evidence of heterogeneity.

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HER2 is a member of the human epidermal growth factor receptor family encoded by a gene located on the long arm of chromosome 17 (17q12–21.32).¹

Around 20% of breast cancers are reported to overexpress HER2 and/or exhibit amplification of the *ERBB2* gene (referred to here as HER2 in line with common practice),² although our experience suggests that, in practice, the rate of HER2 positivity is closer to 15%. HER2 positivity is established as a poor prognostic marker associated with more aggressive disease and decreased survival.^{3–5}

Trastuzumab, a monoclonal antibody targeting HER2, has demonstrated efficacy as a single agent

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or in combination with chemotherapy in patients with HER2-overexpressing primary and metastatic breast cancer.^{6–12} Consequently, treatments that include trastuzumab have become the standard of care for patients with HER2+ early or metastatic disease.^{13–15} As the clinical benefits of trastuzumab are highly associated with HER2 positivity,^{7–12} HER2 testing is recommended at diagnosis of all breast cancers to determine potential eligibility for trastuzumab therapy.¹⁶

Two diagnostic techniques are currently approved for assigning HER2 status in clinical practice: immunohistochemistry (IHC) and *in situ* hybridization (ISH). Whereas IHC uses an antibody to evaluate HER2 protein expression, ISH determines the number of *HER2* copies using a DNA probe coupled to a fluorescent, chromogenic, or silver detection system (ie, FISH, CISH, or SISH), or a combination of CISH and SISH systems (bright-field double ISH (BDISH) or dual-hapten, dual-color ISH (DDISH)). ISH is conducted using a single probe to enumerate *HER2* copies per nucleus only or as a dual-probe technique where hybridization of a chromosome 17 centromere probe (chromosome enumeration probe 17, CEP17) allows determination of the *HER2*:CEP17 ratio. The two-probe approach may be performed as a dual-color technique, with cohybridization of the two probes on the same slide, or as a monochrome assay where each probe is used on sequential slides. The *HER2*:CEP17 ratio is sometimes regarded as a better reflection of *HER2*

amplification status than mean *HER2* copy number, as the latter is also dependent on the mitotic index of the tumor, section thickness, nuclear truncation effects, and abnormal chromosome copy number (aneusomy).¹⁷

US Food and Drug Administration-approved package inserts for HER2 tests originally defined HER2 positivity according to IHC scores of 2+ (weakly positive) or 3+ (positive), mean *HER2* copies per nucleus >4 or *HER2*:CEP17 ratio ≥ 2.0 depending on the type of assay used (Table 1). Patients with IHC 2+/ISH-negative tumors are not eligible for trastuzumab treatment to date. However, 2007 guidelines from the American Society of Clinical Oncology and College of American Pathologists raised the thresholds for HER2 positivity and introduced equivocal ranges for IHC and FISH results.² The guidelines state that an equivocal result should prompt additional testing, but the authors have since emphasized that trastuzumab should not be withheld from patients with equivocal scores that would be considered positive under the original Food and Drug Administration cutoffs.¹⁸ American Society of Clinical Oncology and College of American Pathologists recommended either IHC or FISH as an initial assay, but others prefer FISH as the primary test, arguing that the accuracy of IHC testing is limited by technical considerations and the inherent subjectivity of scoring.¹⁹

Although the American Society of Clinical Oncology and College of American Pathologists algorithms

Table 1 Food and Drug Administration⁸⁹ and American Society of Clinical Oncology/College of American Pathologists² cutoffs for HER2 status determination by IHC or FISH

	IHC		FISH			
	FDA	ASCO/CAP	HER2 copies, average per nucleus		HER2:CEP17 ratio	
			FDA	ASCO/CAP	FDA	ASCO/CAP
Negative	0–1 +	0–1 +	≤ 4.0	<4.0	<2.0	<1.8
Equivocal	—	2+ (nonuniform or weak complete membrane staining in $\geq 10\%$ tumor cells, or intense, complete membrane staining in $\leq 30\%$ invasive tumor cells)	—	4.0–6.0	—	1.8–2.2
Positive	2+ (weak-to-moderate complete membrane staining in $>10\%$ of tumor cells), 3+ (strong complete membrane staining in $>10\%$ of tumor cells). Patients with IHC 2+/ISH– tumors are not eligible for trastuzumab treatment to date	3+ (uniform intense membrane staining of $>30\%$ invasive tumor cells)	>4.0	>6.0	≥ 2.0	>2.2

Abbreviations: ASCO/CAP, American Society of Clinical Oncology/College of American Pathologists; CEP17, chromosome enumeration probe 17; IHC, immunohistochemistry; FDA, Food and Drug Administration; FISH, fluorescence *in situ* hybridization.

provide clear guidance for HER2 assessment, interpretation of test results can be rendered difficult by complexities of breast tumor biology, such as aneusomy of chromosome 17 (including polysomy or monosomy), colocalization of the CEP17 and the *HER2* signals, and intratumoral genetic heterogeneity.²⁰ As monosomy and colocalization are rare, this review focuses on polysomy of chromosome 17 and genetic heterogeneity. We summarize the evidence relating to these issues and discuss its relevance for the interpretation of HER2 tests and trastuzumab eligibility in clinical practice (summarized in Figures 1 and 2).

Polysomy 17

Gene amplification refers to an increase in the copy number of a specific chromosomal region and is commonly linked to overexpression of the affected genes.²¹ Well over 90% of HER2-overexpressing breast tumors show focal gains involving the *HER2* locus.²² In contrast, polysomy is defined by the presence of extra copies of one or more whole chromosomes. Although polysomy of chromosome 17 provides an alternative mechanism for increasing *HER2* gene dosage, its effect on HER2 expression, other clinicopathologic variables, prognosis, and treatment response is not well established. Nonetheless, these are important issues, as elevated

CEP17 count ('polysomy') is frequently reported in breast cancer²³ and is a major reason for discordant ISH status, depending on whether mean *HER2* copy number or *HER2*:CEP17 ratio is used.^{24,25} Some have argued that correction for chromosome 17 copy number is essential for accurately defining HER status,^{19,24} but others have questioned this view.^{26,27} Currently, there is no consensus on whether or how elevated CEP17 count ('polysomy') should be integrated into HER2 test interpretation.

Defining Polysomy 17

In the absence of cytogenetic analysis, the number of CEP17 signals observed by ISH has generally been used as a surrogate for chromosome 17 copy number (Figures 3 and 4). However, the appropriate CEP17 copy cutoff to define elevated CEP17 status ('polysomy') is not obvious, as even truly disomic samples may deviate from the expected value of 2 because of sectioning artifacts, the presence of G₂ cells, and chromosomal instability.²⁸ Several definitions have been proposed, and none is accepted as standard,^{28–51} but a mean of ≥ 3 CEP17 signals per nucleus is a commonly adopted threshold,² with most studies using this definition, including large cohort (≥ 100 cases) studies.

Figure 1 Consensus algorithm for human epidermal growth factor receptor 2 (HER2) status determination in breast cancer samples with apparent polysomy 17 (increased chromosome enumeration probe 17 (CEP17) count). IHC, immunohistochemistry; ISH, *in situ* hybridization.

Figure 2 Consensus algorithm for human epidermal growth factor receptor 2 (HER2) status determination in breast cancer samples showing genetic heterogeneity. **Match with IHC stained slides.

Figure 3 Breast tumor cells analyzed by dual-color silver *in situ* hybridization, using human epidermal growth factor receptor 2 (HER2) (black) and chromosome enumeration probe 17 (CEP17) (red) probes, showing apparent polysomy 17 (increased CEP17 count) with HER2 amplification (a), increased CEP17 count without HER2 amplification (b), and coamplification of HER2 and CEP17 (c), with strong HER2 overexpression (inset: immunohistochemistry (IHC) 3+). Monosomy with HER2 amplification (d) is demonstrated by fluorescent *in situ* hybridization, using HER2 (red) and CEP17 (green) probes.

Prevalence of Polysomy 17 in Breast Cancer

Defined by the relevant authors as an elevated CEP17 count, 'polysomy 17' is a common finding

in invasive breast cancer series.^{28–51} Using the definition of ≥ 3 CEP17 copies per cell, reported prevalence rates range between 3% and 46% (Table 2).^{30–32,34,36–38,41–47,50–51} The wide variation

Figure 4 Schematic diagrams showing nuclei with (a) disomy, human epidermal growth factor receptor 2 (*HER2*)-unamplified; (b) disomy, *HER2*-amplified; (c) polysomy, *HER2*-unamplified; (d) *HER2*/chromosome enumeration probe 17 (CEP17) coamplification.

Table 2 Prevalence of elevated CEP17 count ('polysomy') in breast cancer series

Study	Sample	Definition	Prevalence (%)
Bose <i>et al</i> ³⁰	Breast cancer with equivocal or weakly positive ($n = 52$) or negative ($n = 22$) IHC result	CEP17 copy ≥ 3	34
Cuadros <i>et al</i> ³¹	Breast carcinoma ($n = 462$)	Mean CEP17 ≥ 3	38
Downs-Kelly <i>et al</i> ³²	Consecutive breast cancer cases ($n = 727$)	Mean CEP17 ≥ 3 per cell	8
Hofmann <i>et al</i> ³⁴	Metastatic breast cancer with IHC 3+ or <i>HER2</i> :CEP17 ≥ 2.0 ($n = 95$)	Mean CEP17 ≥ 3 /cell	27
Kokate <i>et al</i> ³⁶	Invasive breast cancer with nonequivocal FISH ratio ($n = 390$)	Mean CEP17 > 3	7
Krishnamurti <i>et al</i> ³⁷	Invasive breast carcinoma ($n = 365$)	Mean CEP17 ≥ 3 /cell	23
Merola <i>et al</i> ³⁸	Breast carcinoma, IHC 2+ ($n = 343$)	Mean CEP17 copy > 3	46
Petroni <i>et al</i> ⁵²	Invasive ductal breast cancer ($n = 647$)	Mean CEP17 ≥ 3	27
Salido <i>et al</i> ⁴¹	Consecutive breast cancer cases, IHC 0–3+ ($n = 50$) or IHC 2+/3+ ($n = 125$)	Mean CEP17 ≥ 3 per cell	13
Takehisa <i>et al</i> ⁴²	Presurgery fine-needle aspiration cytology samples from primary breast cancer patients ($n = 40$)	> 3 CEP17 signals in $> 20\%$ of nuclei	43
Torrissi <i>et al</i> ⁴³	Breast cancer with IHC 0–2+ ($n = 415$) or IHC 3+ ($n = 42$)	CEP17 copy ≥ 3	17
Tsakamoto <i>et al</i> ⁴⁴	Invasive ductal carcinoma ($n = 113$)	$> 20\%$ nuclei with CEP17 > 3	37
Tubbs <i>et al</i> ⁴⁵	Primary breast carcinomas ($n = 742$)	CEP17 count ≥ 3 in 80% of cells	3
Vanden Bempt <i>et al</i> ⁴⁶	Primary invasive breast cancer, IHC 2+/3+ ($n = 171$) or IHC 0/1+ ($n = 55$)	Mean CEP17 ≥ 3 per cell	46
Varshney <i>et al</i> ⁴⁷	Invasive breast cancer ($n = 687$)	Mean CEP17 copy ≥ 3	10
Żaczek <i>et al</i> ⁵⁰	Stage I–III breast cancer ($n = 149$)	≥ 3 CEP17 signals in $> 30\%$ of nuclei	40
Zhu <i>et al</i> ⁵¹	Invasive breast cancer ($n = 481$)	Mean CEP17 ≥ 3 per cell	28

Abbreviations: CEP17, chromosome enumeration probe 17; IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

in estimates may be attributable both to differences in tumor characteristics and the CEP17 cutoff adopted.

Polysomy 17 and Clinicopathologic Features

Along with *HER2*, chromosome 17 contains several other genes implicated in tumorigenesis (eg, *BRCA1*, *TOP2A*, *TP53*),²³ raising the possibility that polysomy might influence clinicopathologic and prognostic variables due to altered expression of these growth-regulatory genes/proteins. Several studies have linked elevated CEP17 count ('polysomy') with unfavorable pathologic features compared with disomic tumors. In unselected breast carcinoma series, polysomy/aneusomy 17 have been associated with increased proliferative activity and greater Nottingham Prognostic Index (NPI) score, although data regarding nodal involvement, histologic grade, and hormone receptor status are conflicting.^{42,49,52} In contrast, a prospective analysis restricted to IHC 2+/3+ tumors ($n=147$) found a significant association between elevated CEP17 count ('polysomy') (defined as CEP17 ≥ 3) and node positivity, but not histologic grade or hormone receptor status.⁴¹ These studies found no significant impact of polysomy on survival, although a trend toward greater relapse was reported for polysomic, IHC 2+/3+ tumors.^{41,42,49}

There is evidence that elevated CEP17 count ('polysomy') might confer a more aggressive phenotype in the absence of specific *HER2* amplification. In a comparison of breast carcinomas with normal *HER2*:CEP17 ratios, elevated CEP17 count ('polysomy') (CEP17 ≥ 3 ; $n=44$) was associated with higher nuclear and histologic grade, greater mitotic activity, poorer NPI score, and estrogen receptor (ER) negativity compared with non-polysomic tumors ($n=36$).³⁷ Indeed, the pathologic features of polysomic *HER2*-nonamplified tumors showed a trend toward those with specific *HER2* amplification ($n=35$). In contrast, Vanden Bempt *et al*⁴⁶ found no significant differences between polysomic (CEP17 ≥ 3) and non-polysomic primary breast cancers (both groups without *HER2* amplification) in clinicopathologic variables, including histologic grade, tumor size, NPI, nodal status, or hormone receptor positivity. Consistent with another study,⁴³ polysomic, *HER2*-nonamplified tumors had more favorable pathologic features compared with *HER2*-amplified tumors. Although patients with polysomic, nonamplified tumors had superior disease-free survival (DFS) compared with *HER2*-amplified tumors ($P=0.031$), there was a trend toward decreased survival with polysomic *versus* non-polysomic tumors in the absence of *HER2* amplification ($P=0.056$).⁴⁶

Despite numerous inconsistencies between the studies, these data provide some indications that elevated CEP17 count ('polysomy') may be associated

with unfavorable clinicopathologic variables and poorer prognosis, albeit to a lesser extent than *HER2* amplification.

Polysomy 17 and *HER2* Overexpression

Gene amplification is thought to be the primary genetic mechanism underlying *HER2* overexpression, but increased gene dosage secondary to polysomy 17 could also contribute to increased protein levels. Several studies have correlated elevated CEP17 count ('polysomy') with increased IHC score in tumors without specific *HER2* amplification.^{29,35,47,53} For example, Hyun *et al*³⁵ reported a significantly higher incidence of elevated CEP17 count ('polysomy') (CEP17 >2.25) in tumors with IHC 2+/3+ *versus* IHC 0/1+ results (40.8% *versus* 12.3%, $P<0.001$). In the absence of *HER2* amplification, elevated CEP17 count ('polysomy') has been commonly associated with equivocal IHC results (IHC 2+).^{30,38,41,46,52} In the series of Vanden Bempt and co-workers,⁴⁶ 46 of 62 polysomic, *HER2*-nonamplified (74.2%) tumors tested IHC 2+, but none were IHC 3+. However, other studies have associated elevated CEP17 count ('polysomy'), particularly high CEP17 counts (CEP17 ≥ 4), with IHC 3+ staining.^{25,47,52} In one series, 17 of 150 (9.8%) polysomic, *HER2*-nonamplified tumors showed positive (3+) immunohistochemistry staining compared with 6 of 247 (1.5%) eusomic, *HER2*-nonamplified cases.⁵²

Others have found no evidence that elevated CEP17 count ('polysomy') contributes to *HER2* overexpression. In a series of 584 breast cancers with normal *HER2*:CEP17 ratios, there was no significant difference in *HER2* protein expression (IHC 0/1+/2+ *vs* 3+) between tumors with normal *versus* increased CEP17 count (≤ 2.25 *versus* >2.25 per cell).²⁴ In another study of 56 *HER2*-nonamplified breast cancers showing elevated CEP17 count ('polysomy') (CEP17 ≥ 2.1), IHC was mostly 0 or 1+ (70%), with some 2+ (27%) and a minority scoring 3+ (4%).³²

Taken together, the balance of evidence suggests that elevated CEP17 count ('polysomy') may have a role in overexpression of *HER2*, particularly in tumors with IHC 2+ scores. Interestingly, there is little evidence that polysomy 17 upregulates *HER2* mRNA,^{32,54} a fact that has led some to propose post-translational mechanisms to account for *HER2* protein increases.³²

We hypothesize that the regulation of *HER2* expression may differ between cases showing selective *HER2* amplification, segmental *HER2*/CEP17 (or other) coamplifications, or true polysomy. The biological impact of such changes in genome organization may be rather heterogeneous, in line with contradictory reports of the correlation between apparent chromosome 17 polysomy and *HER2* overexpression.²⁴⁻²⁶ As a result, it may be

important to obtain additional information on the *HER2* locus and its expression profile at the mRNA and protein levels, going beyond the limited information that is provided by FISH using only two probes.

Polysomy 17 and Response to Anti-HER2 Therapy

The potential association between elevated CEP17 count ('polysomy') and *HER2* expression raises the question of whether increased chromosome 17 copy number influences trastuzumab response. This is particularly important in the context of patients, with a polysomy-associated IHC 3+ score but negative FISH ratio, who might be assumed to have a 'false-positive' IHC result and therefore be considered ineligible for trastuzumab. To address this question, Hofmann *et al*³⁴ performed a retrospective analysis of trastuzumab response by CEP17 status using data from a Phase II study of first-line trastuzumab monotherapy in 105 patients with *HER2* + (IHC 3+ or FISH-amplified) metastatic breast cancer. Elevated CEP17 count ('polysomy') (CEP17 ≥ 3) was observed in 26 patients (27%), of whom six responded to trastuzumab. All six had documented IHC 3+ scores, while four had a *HER2*:CEP17 ratio ≥ 2.0 . The remaining two responders had negative FISH ratios (< 2.0) and elevated mean *HER2* copy number owing to polysomy. This supports the concept that some patients with *HER2* overexpression due to polysomy rather than amplification will respond to trastuzumab and suggests that IHC 3+ tumors should be considered *HER2* + irrespective of the FISH result.

Further data supporting the trastuzumab sensitivity of polysomic tumors emerged from the CALGB 150002 study,⁵⁵ a correlative companion to the pivotal 9840 trial, which randomized a locally tested, *HER2*-negative metastatic breast cancer cohort to receive paclitaxel with or without trastuzumab.⁵⁶ Of 192 tumors with a normal *HER2*:CEP17 ratio (< 2.0) on central testing, 38 (20%) were found to have elevated CEP17 count ('polysomy') (CEP17 ≥ 2.2). The vast majority of these polysomic tumors (34/37; 92%) tested negative/equivocal for *HER2* overexpression (IHC 0/1+/2+). In contrast to the *HER2*-negative cohort as a whole, the subgroup of patients with *HER2*-nonamplified, polysomic tumors derived significant benefit in terms of overall response rate with paclitaxel plus trastuzumab *versus* paclitaxel alone (63% vs 26%, $P = 0.048$). These data suggest that trastuzumab may be efficacious in polysomic, *HER2*-nonamplified tumors, even in the absence of an unequivocally positive IHC result. In contrast, a subgroup analysis of the Phase III EGF30001 trial revealed no significant benefit with first-line lapatinib plus paclitaxel *versus* paclitaxel alone in patients with *HER2*-nonamplified, polysomic metastatic breast cancer.⁵⁷

The impact of elevated CEP17 count ('polysomy') has also been evaluated in a retrospective analysis of the Phase III *HER*ceptin Adjuvant (HERA) trial, which randomized women with *HER2* + early breast cancer who had completed locoregional therapy and at least four cycles of (neo)adjuvant chemotherapy, to 1 or 2 years of trastuzumab or to undergo observation alone.^{9,58} Among patients with a positive FISH ratio, 883 of 2030 (43%) had elevated CEP17 count ('polysomy') (≥ 3 CEP17 signals in $\geq 30\%$ of nuclei). Importantly, in this *HER2* + population (centrally confirmed IHC 3+ or *HER2*:CEP17 ≥ 2.0), trastuzumab maintained consistent DFS benefit across the subgroups of patients with or without elevated CEP17 count ('polysomy'). These results suggest that the efficacy of adjuvant trastuzumab in patients with *HER2* + early breast cancer, assessed according to standard criteria, is unaffected by elevated CEP17 count. Only 41 patients were diagnosed as *HER2* + despite a FISH ratio < 2.0 ; this number is too small to determine whether adjuvant trastuzumab was beneficial. Elevated CEP17 count ('polysomy') might also predict response to other therapies. For example, increased copy number of the gene for topoisomerase II, via gene amplification or polysomy 17, has been associated with sensitivity to anthracycline-based neoadjuvant therapy.⁵⁹

True Polysomy or CEP17 Amplification?

Standard ISH analyses generally assume that the number of CEP17 signals corresponds to the number of chromosome 17 copies. However, concurrent evaluation of several chromosome 17 genes using multiple-probe FISH or multiplex ligation-dependent probe amplification (MLPA) suggests that focal amplifications encompassing the centromere are the usual reason for high-level increases in CEP17 signals.^{17,60,61} Of 171 breast tumors with apparent polysomy (CEP17 > 2.6), only 24 cases (14%) showed the equivalent increases in copy numbers of CEP17, *HER2*, *SMS*, *RARA*, and *TP53* that would result from whole chromosomal gains.¹⁷ Similarly, MLPA analysis of 17 chromosome 17 loci identified no cases of true polysomy among 111 breast cancers, although 19% had shown CEP17 increases by CISH.⁶⁰

Molecular karyotyping studies have confirmed the rarity of true polysomy 17 in breast cancer. For example, Marchiò *et al*⁶² used array-comparative genomic hybridization (aCGH) to make a detailed assessment of chromosome 17 status in 18 breast tumors with apparent polysomy 17 (CEP17 ≥ 3). Strikingly, only one case demonstrated gains of both the short and long arms of the chromosome, which is a requirement for a true polysomic state. The remainder showed gains of 17q with centromeric involvement ($n = 11$), 17q gain without centromeric involvement ($n = 1$), or focal centromeric amplification

($n=5$). Similarly, an aCGH analysis of 97 breast cancer specimens found no cases of true polysomy, despite the inclusion of two cases with elevated CEP17 copy number.⁶³ Single-nucleotide polymorphism karyotyping has also shown that true polysomy 17 is uncommon.²⁵

Collectively, these studies show that elevated CEP17 copy number is not a useful surrogate for polysomy 17 and that most cases previously assigned 'polysomic' status actually represent focal pericentromeric gains.

Authors' Consensus Recommendations

The erroneous assumption that the number of CEP17 signals observed by ISH corresponds to the number of chromosome 17 copies may explain many of the inconsistencies in the polysomy literature. Tumors with abnormal CEP17 copy number may, in fact, represent a heterogeneous group with complex chromosomal gains and losses, whose clinicopathologic features may be dependent on the specific genetic changes present. In the research setting, we recommend the use of more sophisticated techniques (eg, aCGH) to define more accurately the chromosome 17 status of a tumor, and to help clarify the biologic significance of these genetic aberrations in patients with breast cancer.

In the meantime, these findings have practical implications for interpretation of HER2 tests in clinical practice (Figure 1).^{64,65} Importantly, use of the *HER2*:CEP17 ratio in isolation may be misleading in cases with CEP17 gains or losses, which would lead to under- and overestimation of *HER2* amplification, respectively. We therefore recommend that raw ISH data (cell count, absolute *HER2*, and CEP17 signals) be included in pathology reports to assist interpretation.⁶¹ In cases with elevated CEP17 count, it is advisable to use mean *HER2* copy number (≥ 6 copies) for defining HER2 status. This approach is supported by the high concordance between IHC staining intensity and mean copy number in the HERA trial⁶⁶ and between IHC and single-probe SISH results (98.3%) in a recent analysis of more than 1200 breast cancers.⁶⁷ In this latter study, the result of single-probe SISH was more closely correlated than dual-probe SISH with IHC score, among tumors with discordant single- versus dual-probe ISH assessment. Although Bartlett *et al*⁶⁸ in 2008 recommended use of the *HER2*:CEP17 ratio for samples with CEP17 elevation, this study predates the bulk of the data showing that true polysomy is rare. Using patient screening data from the HERA trial, we estimated that the use of mean copy number alongside the ratio would identify an additional 29 patients (0.5% of all FISH tests) for trastuzumab eligibility (J Rüschoff *et al*, personal communication). The same approach being applied to the series of Vanden Bempt *et al*⁴⁶ and Dal Lago *et al*²⁴ would yield an

additional 2.2% and 1.5% of patients as 'HER2+', respectively.

We also note that confirmatory IHC can provide useful supporting information in complex cases.⁶¹ For those patients whose diagnostic picture remains unclear, the use of additional ISH probes for alternative reference genes, such as *RARA* and *SMS*, may help resolve ambiguous HER2 status.¹⁷ Finally, we anticipate an increasing role for molecular karyotyping techniques in supporting HER2 status determination for complex cases.⁶⁹ In a study of 20 samples referred because of discordance, equivocal results, or suspected pericentromeric aneusomy, aCGH was able to resolve HER2 status accurately in all cases.⁷⁰ Notably, 16 cases displayed complex rearrangements of chromosome 17, although one showed true polysomy with low-level *HER2* amplification, and a second tumor was monosomic.

In conclusion, we consider that HER2 status is best defined by evaluating IHC score, mean *HER2* copy number, and *HER2*:CEP17 ratio. Samples scoring IHC 3+ should be scored HER2+ (taking into account well-controlled methods for assessment), regardless of ISH result and elevated CEP17 count ('polysomy'). When the mean *HER2* copy number is ≥ 6 , and the IHC score is 2+, tumors should be assessed as HER2+, irrespective of *HER2*:CEP17 ratio. Such a strategy will help ensure that patients who may benefit from trastuzumab are not denied access to this potentially life-saving therapy.

Genetic heterogeneity

Cancer-associated mutations often exhibit non-uniform distributions among tumor cells.⁷¹ In its broadest sense, '*HER2* genetic heterogeneity' describes the coexistence of multiple tumor-cell subpopulations with distinct *HER2* amplification characteristics within the same individual (Figures 5 and 6). The term encompasses both intratumoral heterogeneity, where *HER2* gene status differs between cells in the same or different regions of a single tumor, and intertumoral heterogeneity, where there is discordance between a primary tumor and its metastases.⁷² Two main distributions of intratumoral heterogeneity have been described: a 'clustered' form characterized by pockets of highly amplified cells, sometimes possessing distinct histologic architecture or grade, and a 'mosaic' form in which distinct subpopulations comingle, although some argue that this mosaic pattern is, in fact, an ISH artifact.⁷²⁻⁷⁴

Genetic heterogeneity is clinically relevant in two major respects. First, heterogeneity could lead to discordant HER2 status between blocks of the same tumor, in core biopsies versus surgical specimens, in primary tumors versus metastases, among results of different laboratories, and between IHC and ISH assays. Second, the potential relevance of

Figure 5 Genetically heterogeneous tumors analyzed by different methods. (a) Immunohistochemistry (IHC) of a genetically heterogeneous breast tumor showing clusters of human epidermal growth factor receptor 2 (HER2)+ cells (brown staining). (b) Double fluorescence *in situ* hybridization/IHC staining showing heterogeneity made up of tumor cells showing both HER2 protein overexpression (green membrane staining) and HER2 gene amplification (red signals). (c) Genetically heterogeneous tumor analyzed using dual-hapten, dual-color *in situ* hybridization (HER2, black staining; chromosome enumeration probe 17, red staining).

Figure 6 Schematic diagrams illustrating breast cancer specimens with (a) human epidermal growth factor receptor 2 (*HER2*)-nonamplified, genetically homogeneous tumor; (b) *HER2*-amplified, genetically homogeneous tumor; (c) genetically heterogeneous tumor with 'mosaic' pattern in which amplified and unamplified clones comingle; (d) genetically heterogeneous tumor with a cohesive clone of amplified cells surrounded by nonamplified cells. Blue polygons represent nuclei with a normal *HER2*:CEP17 ratio (≤ 2.2); red polygons represent nuclei with an elevated *HER2*:CEP17 ratio (> 2.2).

subpopulations of *HER2*-amplified cells within otherwise nonamplified tumors has been a topic of debate, particularly regarding prognostic significance and potential benefit from trastuzumab.

Defining Genetic Heterogeneity

Although genetic heterogeneity has been variously defined in terms of regional differences in *HER2*

amplification,^{75,76} a standardized approach for assessment of nonuniform *HER2* amplification was published in 2009 by a College of American Pathologists Expert Panel.⁷⁴ This guideline defines genetic heterogeneity according to the presence of >5% but <50% of infiltrating tumor cells with a *HER2*:CEP17 ratio >2.2 (or mean *HER2* copy number >6). In heterogeneous cases, the Panel also recommends that the proportion of invasive tumor cells showing *HER2* amplification be reported alongside the mean value for all cells analyzed, with separate analysis of any clusters present.

Although not based on outcomes data, an explicit goal of this guideline was to standardize the definition of genetic heterogeneity to facilitate future study of its clinical relevance. Nonetheless, some have questioned the recommendation for routine reporting on a histopathologic phenomenon for which clinical significance remains poorly defined.⁷⁷ Others have suggested that the upper limit for amplified cells should be increased to 95% so that tumors with mixed populations in which *HER2*-amplified cells predominate (ie, 50–95% cells) are also captured within the definition.⁷⁸ Some researchers have argued that the proportion of amplified cells is not a useful measure of nonuniformity within a tumor.⁷⁹ The College of American Pathologists guideline also introduces an apparent discrepancy in assessment between ISH and IHC methods: whereas homogeneous ISH positivity requires ≥50% amplified cells, *HER2* positivity defined according to IHC requires adequate staining in >10% of cells (Food and Drug Administration cutoff) or >30% of cells (American Society of Clinical Oncology and College of American Pathologists cutoff). As the same tumor-cell populations in heterogeneous tumors show both *HER2* protein overexpression and *HER2* gene amplification (Figure 5), this disparity is liable to introduce discordance between IHC and ISH results.

Alternative definitions of genetic heterogeneity include that from the Italian Society of Human Genetics (SIGU), which recommends that different subclones be identified according to cytogenetic pattern (ie, low, high, or absence of amplification) and FISH ratio reported separately for each

subpopulation present.⁷³ UK guidelines define genetic heterogeneity according to the observation of distinct fields within the tumor showing amplified and nonamplified *HER2*:CEP17 ratios.⁸⁰ Recently, Bartlett and co-workers⁸¹ suggested classifying heterogeneity with amplification as simple, complex, or isolated amplified tumor cells, and provided reporting recommendations for these cases.

Prevalence of Genetic Heterogeneity

Although an early application of the College of American Pathologists definition found a low prevalence of genetic heterogeneity in primary breast carcinomas (40/742, 5%),⁴⁵ more recent studies suggest that a third or more of breast tumors exhibit heterogeneous *HER2* amplification (11–40%; Table 3).^{28,40,79,82–84} Of note, one study reported a far higher rate of genetic heterogeneity when defined by *HER2*:CEP17 ratio (23%) than with mean *HER2* copy number (7%), raising some concerns over the validity of the College of American Pathologists criterion.⁸² In another study, consideration of genetic heterogeneity tripled the proportion of patients with *HER2* abnormalities (ie, amplified and/or heterogeneous, 45%) compared with *HER2* amplification alone (14%).⁸³

Intratumoral heterogeneity is also observed at the level of *HER2* protein overexpression (Figures 5a and b). In the Adjuvant Lapatinib and/or Trastuzumab Treatment Optimization (ALTTO) study, 1091 of 8490 patients (12.85%) had tumors with IHC3+ staining in 1–50% of cells (G Viale, personal communication). Similarly, 3.7% of patients enrolled in the N9831 study had tumors with IHC3+ staining in 10–30% of cells.⁸⁵

Impact of Genetic Heterogeneity on *HER2* Status Determination

Intratumoral genotypic variability could lead to discrepant *HER2* status depending on the region analyzed, and so might introduce sampling errors into *HER2* testing. Brunelli *et al*⁷⁵ estimated that 13% of tumors with low-grade *HER2* amplification

Table 3 Prevalence of College of American Pathologists-defined *HER2* genetic heterogeneity in published studies

Study	Sample	Parameter	Prevalence (%)
Tubbs <i>et al</i> ⁴⁵	Consecutive primary breast carcinomas (n = 742)	<i>HER2</i> :CEP17 ratio	5
Öhlschlegel <i>et al</i> ⁴⁰	Consecutive invasive breast carcinomas (n = 218)	<i>HER2</i> :CEP17 ratio	15
Allison <i>et al</i> ⁸²	Consecutive clinical breast cancers (n = 1329)	<i>HER2</i> :CEP17 ratio	23
		<i>HER2</i> copy number	7
Chang <i>et al</i> ⁷⁹	Consecutive breast carcinomas (n = 2522)	<i>HER2</i> :CEP17 ratio	26
Bartlett <i>et al</i> ⁸³	Breast cancers analyzed in UK routine practice or for a clinical trial (n = 6461)	<i>HER2</i> :CEP17 ratio	34
Murthy <i>et al</i> ²⁸	Invasive ductal carcinoma, IHC 2+ (n = 60)	<i>HER2</i> :CEP17 ratio	40
Seol <i>et al</i> ⁸⁴	Invasive primary breast cancer (n = 96)	<i>HER2</i> :CEP17 ratio	11

Abbreviations: CEP17, chromosome enumeration probe 17; *HER2*, human epidermal growth factor receptor 2; IHC, immunohistochemistry.

exhibit regional genotypic variability that could affect trastuzumab eligibility. Similarly, a recent analysis of 291 nonconsecutive breast cancers showed that final FISH status was dependent on the area selected, section scored, and the number of cells analyzed.⁷³ The authors also caution that focally amplified small populations might be missed in a single, randomly chosen section. In another study of 20 cases with genetic heterogeneity of the primary tumor, axillary node metastases showed loss of the minor *HER2*-amplified clone in 70% of cases. In the remainder, the amplified clone was retained either as a minority population (15%) or as the predominant clone (15%) within the nodes.⁸⁶ Taken together, these studies highlight a need to standardize scoring methodology to ensure the accuracy of ISH testing. Currently, there is wide variation in practice, with different pathologists scoring a whole section, or specific areas selected at random or chosen as 'hot spots'. Furthermore, commercially available ISH kits variously define cutoffs for *HER2* positivity according to examination of a certain number of nuclei (eg, 20 or more nuclei for FISH, dual-color CISH, SISH, BDISH, and DDISH) or a proportion of all cells analyzed (eg, >50% nuclei for single-color CISH).

Prognostic and Predictive Relevance of Genetic Heterogeneity

As intended by the Expert Panel, studies are now exploring the clinical relevance of the College of American Pathologists definition. In a series of 345 core biopsy and resection specimens, Öhlschlegel and colleagues found genetic heterogeneity was significantly associated with elevated CEP17 count ('polysomy 17') (CEP17 >2.25), absence of *HER2* gene clusters (>16 copies per nucleus), high histologic grade, and genomic instability.⁴⁰ However, heterogeneity was not predictable from histologic, immunohistochemical, and DNA cytometric variables.

The prognostic impact of genetic heterogeneity has been assessed in a large cohort including clinical trial participants and patients from routine practice ($n=6461$).⁸³ In this study, there was no significant difference in 5-year DFS in patients with up to 30% of cells with a *HER2*:CEP17 ratio >2.2 compared with those without amplified cells, suggesting that a low proportion of amplified cells may not confer a worse prognosis. However, DFS was reduced among those with 30–100% of amplified cells. Based on an exploratory analysis of cases with 30–50% amplified cells, the investigators suggested that the prognosis was only worsened in the presence of an identifiable amplified clone, although the DFS difference did not reach statistical significance. Another recent study ($n=96$) demonstrated that genetic heterogeneity was independently associated with poorer DFS compared with

tumors with homogeneous *HER2* amplification.⁸⁴ These studies provide the first indications that subpopulations of *HER2*-amplified cells might drive poorer outcome.

From a therapeutic standpoint, the key question is whether genetically heterogeneous tumors respond to trastuzumab. Although the vast majority of such cases do not currently qualify for trastuzumab eligibility based on mean *HER2*:CEP17 ratio or mean *HER2* copy number,⁸² genetically heterogeneous tumors harbor significant populations of *HER2*-amplified cells and so might be sensitive to *HER2*-targeted therapy.⁴⁰ Indeed, this phenomenon might account for the observation of trastuzumab responses in 'HER2-negative' tumors.⁸² In this respect, the results of the ongoing NSABP B-47 trial of adjuvant trastuzumab in *HER2*-low breast cancer are eagerly awaited.

Authors' Consensus Recommendations

One limitation of current guidelines for *HER2* assessment is the difference in approach used to define *HER2* positivity between IHC and ISH assays. In IHC, *HER2*-positivity is defined in terms of the percentage of cells with adequate staining, whereas most ISH techniques define *HER2*-positivity according to mean *HER2* copy number or *HER2*:CEP17 ratio across the analyzed population. Although the College of American Pathologists definition of genetic heterogeneity incorporates information on the proportion of positive cells into the ISH assessment, it also introduces disparity between IHC and ISH methodology in the cutoff for proportion of cells showing *HER2* abnormalities in the sample. Therefore, in heterogeneous cases and to ensure consistency with the Food and Drug Administration cutoff for IHC *HER2*-positivity of 10%, we propose that tumors with >10% *HER2*-amplified cells (after counting 20–60 cells from multiple different fields) should be considered *HER2*+. We recommend that the percentage of amplified cells is included in the pathology report. Tumors containing a cohesive, amplified clone should be classed as *HER2*-amplified (with separate scoring for the clone), whereas tumors with scattered amplified cells should be classified according to the overall mean score (Figure 2). The prevalence of genetic heterogeneity and its potential to introduce sampling error into *HER2* status determination highlight an urgent need to standardize ISH scoring methodology. We underscore the importance of scanning the entire slide to ensure that small pockets of amplified cells are not missed. Ideally, two or more representative fields should be selected and at least 20 randomly selected cells scored within each area, but this might not be feasible in the case of core biopsies containing a small amount of tumor. If there is evidence of heterogeneity, assessment of additional cells and/or fields may be

useful, and at least 60 cells should be counted if there are scattered nuclei with *HER2* amplification. Specifically, additional blocks of the tumor and of lymph-node metastases (if present) should be tested to avoid missing an area of frank *HER2* amplification. We also note the value of bright-field ISH techniques (ie, CISH, SISH, BDISH, and DDISH) in assessment of genetic heterogeneity, as these assays permit evaluation of the entire slide under light microscopy.⁷² Improved FISH techniques⁸⁷ may also reduce the risk of overlooking focal amplified cells due to signal quality and increased DAPI counterstain. In addition, staining of slides can be carried out within 4 hours (J Rüschoff, in preparation). As IHC can also assist in identifying target cells for ISH assessment, a newly developed combined gene-protein assay may be a valuable tool in these cases.⁸⁸

Finally, we highlight the need for outcomes data addressing whether genetically heterogeneous tumors with *HER2*-amplified subclones respond to trastuzumab. Analysis of archival tissue samples from trastuzumab clinical trials may help answer this question and determine whether eligibility

criteria can be expanded to include patients with genetically heterogeneous tumors not currently regarded as *HER2* +. At present, there is no indication for trastuzumab in patients with 1–10% amplified cells. For these patients, we recommend repeating the assay on different tumor blocks or lymph-node metastases. If the percentage remains in the 1–10% range, the case should be considered negative and the proportion of amplified cells should be included in the pathology report, as these patients are most likely to benefit from subsequent re-testing of distant metastases.

Conclusions

Apparent polysomy of chromosome 17 and genetic heterogeneity of *HER2* present major diagnostic challenges when evaluating *HER2* status. In this review, we have made several recommendations based on the literature review and the clinical experience of the authors to assist *HER2* status determination in such complex cases (summarized in Figures 1 and 2, and Table 4). It is our hope that

Table 4 Authors' consensus recommendations

Polysomy 17	Genetic heterogeneity
<ul style="list-style-type: none"> Increases in CEP17 copy number observed by ISH should not be interpreted to indicate polysomy of chromosome 17. If there is evidence of centromeric abnormalities, absolute <i>HER2</i> copy number (mean ≥ 6 copies) is preferable to <i>HER2</i>:CEP17 ratio for defining <i>HER2</i> status. <i>HER2</i> + status should be assigned if any one or more of the following applies: IHC 3+; <i>HER2</i>:CEP17 ratio ≥ 2; or mean <i>HER2</i> copy number ≥ 6. IHC 3+ tumors should be considered <i>HER2</i> + (taking into account well-controlled methods for assessment) irrespective of the FISH result or polysomy 17. When the mean <i>HER2</i> copy number is ≥ 6 and the IHC score is 2+, tumors should be assessed as <i>HER2</i> +, irrespective of <i>HER2</i>:CEP17 ratio. Confirmatory IHC testing, use of additional chromosome 17 ISH probes (eg, <i>SMS</i>, <i>RARA</i>), or molecular karyotyping may assist determination of <i>HER2</i> status in complex cases. Future studies should utilize aCGH or other pangenomic techniques to further evaluate the clinical significance of abnormal chromosome 17 status, including polysomy. 	<ul style="list-style-type: none"> To ensure consistency with the Food and Drug Administration cutoff of 10% for IHC-assessed <i>HER2</i> status, tumors with $> 10\%$ <i>HER2</i>-amplified cells should be considered <i>HER2</i> +. To avoid missing areas of genetic heterogeneity, it is essential that the entire slide be scanned before selecting areas for analysis. Sufficient representative fields should be analyzed (20 randomly selected cells within each area), with scoring of additional fields and/or cells if there is evidence of heterogeneity. Specifically, additional blocks of the tumor and of lymph-node metastases (if present) should be tested to avoid missing an area of frank <i>HER2</i> amplification. Bright-field ISH techniques (ie, CISH, SISH, BDISH, and DDISH) permit evaluation of the entire slide under light microscopy and so may facilitate evaluation of heterogeneous cases. IHC can also assist identification of target cells for ISH assessment. If a tumor exhibits a cohesive clone of amplified cells, the tumor should be classified as amplified and the amplified clone scored separately from the rest of the tumor. If there are scattered, single amplified cells throughout the tumor, the average <i>HER2</i>:CEP17 score from at least 60 cells (minimum) should be used to classify the case as amplified or nonamplified. For patients with 1–10% <i>HER2</i>-amplified cells, ISH should be repeated on different tumor blocks or on nodal metastases, if available. If the percentage remains in the 1–10% range, the case should be considered negative and the proportion of amplified cells should be included in the pathology report, as these patients are most likely to benefit from subsequent re-testing of metastases. Future studies should evaluate the potential for trastuzumab response in tumors with <i>HER2</i>-amplified subclones not meeting the current criteria for <i>HER2</i>-positivity.

Abbreviations: aCGH, array-comparative genomic hybridization; BDISH, bright-field double *in situ* hybridization; CEP17, chromosome enumeration probe 17; CISH, chromogenic *in situ* hybridization; DDISH, dual-hapten, dual-color *in situ* hybridization; *HER2*, human epidermal growth factor receptor 2; IHC, immunohistochemistry; ISH, *in situ* hybridization; FISH, fluorescence *in situ* hybridization; SISH, silver *in situ* hybridization.

these issues will be further addressed in the next evolution of HER2 testing guidelines to help optimize care for patients with HER2+ breast cancer.

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