‘Genetic heterogeneity’ in *HER2/neu* testing by fluorescence *in situ* hybridization: a study of 2522 cases

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Amplification for the *ERBB2* oncogene encoding the *HER2/neu* protein (*HER2*) is of predictive and prognostic importance in breast carcinoma. Fluorescence *in situ* hybridization (FISH) is a widely accepted method for determining *HER2* amplification status. A *HER2*-amplified tumor is defined as having a ratio of *HER2* signals to chromosome 17 centromeric probe signals (*HER2/CEP17* ratio) exceeding 2.2. However, the presence of scattered cells demonstrating *HER2* amplification is of unclear significance. A 2009 panel guideline defined a tumor with ‘genetic heterogeneity’ as having at least 5% but fewer than 50% of (non-clustered) tumor nuclei with a ratio >2.2. The study objective was to examine the statistical distribution of breast tumors tested by FISH for *HER2* amplification, after implementation of this 2009 guideline. We identified 2522 consecutive breast carcinoma cases (2009–2011) tested for *HER2* amplification. All cases were tested by FISH using a standard clinical protocol, adhering to established guidelines. For each case, data on cell counts were retrieved electronically. Each tumor was compared with a theoretical normal distribution by quantile–quantile analysis. Of 2522 FISH tests for *HER2*, 1900 (75%) were non-amplified, 394 (16%) were amplified, and 228 (9%) were *HER2*-equivocal. A total of 666 (26%) had ‘genetic heterogeneity.’ Among these ‘genetically heterogeneous’ cases, the ratio was non-amplified in 430 (64.5%), amplified in 24 (4%), and equivocal in 212 (31.5%). The amplified subpopulation in ‘genetically heterogeneous’ tumors was larger if the overall ratio was close to 2.2. However, the percentage of nuclei >2.2 in a ‘genetically heterogeneous’ tumor was not informative of the underlying tumor-cell distribution. We conclude that the proportion of *HER2*-amplified nuclei within a tumor does not contribute information independent of the actual *HER2/CEP17* ratio. Reassessment of the definition of ‘genetic heterogeneity’ in *HER2* testing is warranted.

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Human epidermal growth factor receptor 2 (*HER2*) is a membrane tyrosine kinase encoded by the *ERBB2* oncogene.¹ *HER2* is commonly overexpressed or amplified in invasive breast carcinomas, a finding that correlates to high tumor aggressiveness and higher response rates to both chemotherapy (eg, doxorubicin) and targeted anti-*HER2* therapy (eg, trastuzumab, lapatinib).^{1–5} Both immunohistochemistry and *in situ* hybridization are accepted methods

of determining clinical *HER2* status.^{6–8} There is no universal agreement upon a ‘gold-standard’ method; however, *in situ* hybridization is considered to be reliable and reproducible when objective criteria are applied.^{8–10} In current practice, *in situ* hybridization is used either as a primary method of *HER2* determination,¹⁰ or as an additional method to resolve cases that are equivocal by immunohistochemistry.⁹

Establishing a definition for *HER2* gene amplification in breast carcinoma has been an ongoing challenge. *HER2* determination by fluorescence *in situ* hybridization (FISH) requires the counting of *HER2* probe signals and, in most cases, of a chromosome 17 centromeric probe (*CEP17*). At the time of the original trastuzumab trials, tumors with a *HER2/CEP17* ratio of >2.0 by *in situ* hybridization were considered *HER2*-amplified.⁸ Further analysis

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showed that testing discordances arose up to 20% of the time, attributed to statistical variability near the clinical threshold.¹¹ Under a widely accepted guideline,⁹ issued by a joint College of American Pathologists and American Society of Clinical Oncology (CAP/ASCO) panel in 2007, an equivocal interval (*HER2/CEP17* ratio of 1.8–2.2) is used to address this source of variability.

Not addressed by the 2007 CAP/ASCO guideline is another important source of uncertainty: cases that appear to demonstrate intratumoral heterogeneity for *HER2* gene amplification. These cases are challenging because they raise the possibility of a subpopulation of *HER2*-amplified tumor cells—a phenomenon thought to be uncommon but potentially significant.^{10,12–15} However, it is difficult to determine which tumors are heterogeneous, and therefore to determine the clinical significance of intratumoral heterogeneity. To facilitate the prospective collection of these data, the CAP/ASCO panel issued a supplemental guideline in 2009 to define tumors that are 'genetically heterogeneous.'¹⁶ Based on expert opinion but not on clinical evidence, 'genetically heterogeneous' tumors were defined as those with at least 5% but fewer than 50% of nuclei having a *HER2/CEP17* ratio >2.2. The objective of this study was to review the *HER2* FISH testing data available since the implementation of the CAP/ASCO 2009 guideline. Using a descriptive-statistical approach, we evaluate the extent to which the 2009 CAP/ASCO definition of 'genetic heterogeneity' identifies non-uniform tumor populations.

Materials and methods

Clinical Case Selection

Consecutive cases of breast carcinoma ($N=2522$) tested for *HER2* gene amplification by FISH, from April 2009 to June 2011 (inclusive), were identified in accordance with the procedures approved by the institutional research ethics board. These cases represent output from a regional reference center conducting both primary assessment (of biopsies) by FISH, and reflex-testing for (excisional) cases that were *HER2*-equivocal (or unsatisfactory) by immunohistochemistry. Because the objective of the study was to assess heterogeneity in gene amplification specifically, cases were not analyzed with respect to immunohistochemical staining.

FISH Interpretation

Clinical FISH processing and scoring at our institution follows a highly standardized protocol. Hybridization is performed using *HER2/neu* (*nuc ish* 17q11.2–q12) and *CEP17* (*nuc ish* 17p11.1–q11.1) probes from Vysis (Des Plaines, IL, USA). All cases were scored by the same technologists (either JM, MW; or both in equivocal cases) with appropriate

pathologist guidance, in accordance to CAP/ASCO guidelines.^{9,16} Images from all cases were reviewed by the signing pathologist to ensure representative scoring of the invasive component. All tumor areas on the slide were scanned to ensure capture of amplified subpopulations. Sporadic nuclei found to have single *CEP17* signals were considered artifactually truncated, and not included in counting. Per guidelines, clusters of amplified cells were scored separately.

For both biopsies and excisions, at least 20 nuclei were counted (mean of 42, ranging from 20 to 120), with more nuclei counted in equivocal cases. As per the current ASCO/CAP guidelines, a *HER2/CEP17* ratio was considered negative if <1.8; equivocal if ≥ 1.8 but ≤ 2.2 ; and positive if >2.2.⁹ 'Genetic heterogeneity' was defined as having at least 5% but fewer than 50% of non-clustered infiltrative tumor cells with a ratio higher than 2.2.¹⁶

Electronic Data Retrieval and Analysis

Electronic laboratory worksheets were opened by an automated search, and the following data elements were retrieved: number of nuclei counted, mean number of *HER2* and *CEP17* signals, and the mean *HER2/CEP17* ratio. In addition, the *HER2/CEP17* ratio of each cell nucleus was retrieved, and quantile–quantile (Q–Q) analysis was performed using an automated script (written in Python; <http://www.python.org>) as follows. A sorted rank-list of actual *HER2/CEP17* values was retrieved from the laboratory worksheet. The mean *HER2/CEP17* ratio and standard deviation of the actual data were determined and used to generate a matched sorted rank-list of theoretical values conforming to a normal distribution. To estimate the closeness-of-fit of the actual data to a normal distribution, least-squares regression analysis was performed on the paired sorted rank-lists, and the correlation coefficient (R^2) was determined using standard statistical methods.

Results

Prevalence of *HER2* Amplification and 'Genetic Heterogeneity' ($N=2522$)

The *HER2* amplification results of all cases evaluated, as defined by *HER2/CEP17* ratio, are summarized in Table 1 (left). Of 2522 consecutive cases, 394 (16%) were *HER2*-amplified (ratio >2.2), and 1900 (75%) were *HER2*-non-amplified (ratio <1.8). The *HER2/CEP17* ratio fell within the equivocal range (1.8–2.2) in 228 (9%) cases.

As shown in Table 1 (right), 666 cases were classified as 'genetically heterogeneous' by the CAP/ASCO (2009) guideline (26% of all cases). The majority of 'genetically heterogeneous' cases was *HER2*-non-amplified (430/666, 64.5%), and few were *HER2*-amplified (24/666, 4%). Although cases classified as

Table 1 Prevalence of *HER2* gene amplification and 'genetic heterogeneity' ($N = 2522$)

<i>HER2</i> amplification status (based on ratio)		Breakdown of 'genetically heterogeneous' ^a cases	
<i>All cases</i>	2522	<i>'Genetically heterogeneous' cases</i>	666 (26% of all cases)
Amplified (>2.2)	394 (16%)	Amplified (>2.2)	24 ^b (4%)
Equivocal (1.8–2.2)	228 (9%)	Equivocal (1.8–2.2)	212 ^b (31.5%)
Non-amplified (<1.8)	1900 (75%)	Non-amplified (<1.8)	430 ^b (64.5%)

^aGenetically heterogeneous, defined as having $\geq 5\%$ and $<50\%$ amplified tumor cells (CAP/ASCO 2009).

^bGenetically heterogeneous cases comprise 24/394 (6%) of amplified, 212/228 (93%) of equivocal, and 430/1900 (23%) of non-amplified cases.

'genetically heterogeneous' with an equivocal *HER2/CEP17* ratio were in the minority (212/666, 31.5%), among all *HER2*-equivocal cases ($N = 228$), the vast majority were 'genetically heterogeneous' (212, 93%). 'Genetically heterogeneous' cases comprised 24/430 (6%) of amplified, 430/1900 (23%) of non-amplified cases.

'Genetic Heterogeneity' Increases with *HER2/CEP17* Ratio as a Continuous Function

Given that 'genetic heterogeneity' is defined by the percentage of tumor cells with a ratio >2.2 (ie, the percentage of amplified cells), the relation between this percentage and *HER2/CEP17* ratio was evaluated graphically (Figure 1). Examination of the range of values shows that both *HER2/CEP17* ratio and the percentage of amplified cells behave like continuous variables. The percentage of amplified cells was plotted on the ordinate against *HER2/CEP17* ratio, showing that it increases as a continuous function. Within the 'genetically heterogeneous' range, this increase approximates a straight line.

The overall shape of the plot closely resembles the probability of any random variable—including those that are normally distributed—having a value above a given threshold. To illustrate this, the actual values in Figure 1 can be compared with the probability of representative normally distributed populations having values >2.2 . The theoretical curve shown in Figure 1 (solid line) was constructed by taking a normal distribution for each data point having the same mean *HER2/CEP17* ratio and a s.d. of 0.3 (comparable to the average standard deviation of the actual cases), and calculating the cumulative probability of *HER2/CEP17* >2.2 . As expected, when *HER2/CEP17* ratio has a value of 2.2 (the amplification threshold), the percentage of amplified cells is $\sim 50\%$. This finding does not depend on the actual distribution of *HER2*-amplified cells within the tumor (see below).

Both Genetically Uniform and Non-Uniform Tumors may have a High Percentage of *HER2*-Amplified Cells

Tumors in which *HER2* is amplified according to a normal distribution (ie, genetically uniform tumors) could be identified either by inspecting a frequency

Figure 1 'Genetic heterogeneity' increases with *HER2/CEP17* as a continuous function. The percentage of tumor cells with a *HER2/CEP17* ratio >2.2 is plotted against *HER2/CEP17* ratio (hollow circles). For comparison, a theoretical curve (solid line) is plotted by calculating the probabilities of *HER2/CEP17* >2.2 for a representative normally distributed population (mean = *HER2/CEP17* ratio, s.d. = 0.3). When *HER2/CEP17* ratio has a value of 2.2 (the amplification threshold), the percentage of amplified cells is $\sim 50\%$ (dotted lines) ($N = 2520$; for clarity two data points having *HER2/CEP17* ratio >20 are omitted).

histogram of *HER2/CEP17* ratio (Figure 2a), or by inspecting the plot of actual ranked values against a theoretical normal distribution matched with respect to both mean and s.d. (Q–Q plot, Figure 2b). As shown by the illustrative example in Figure 2b, genetically uniform tumors correspond to Q–Q plots that are well fitted by linear regression (ie, R^2 approaching 1.0).

A contrasting example of a genetically non-uniform tumor is illustrated in Figures 3 and 4. Such tumors could sometimes be identified visually during manual counting of *HER2* and *CEP17* signals under the fluorescence microscope (Figure 3). Within

Figure 2 Example of a tumor with a uniform distribution having a high percentage of *HER2*-amplified cells. Example of a case with *HER2/CEP17* ratio = 2.14, with 42% of cells having a *HER2/CEP17* ratio > 2.2. As seen in the histogram (a), the *HER2/CEP17* appears to be uniformly distributed around the mean. Quantile–quantile analysis (b) shows that the tumor population closely resembles a normal distribution (R^2 of the actual-vs-theoretical plot = 0.95).

Figure 3 Microscopic appearance (FISH) of a tumor with a non-uniform population. Representative field (FISH microscopy, with orange *HER2* signals, green *CEP17* signals, and DAPI counterstain) of a case with *HER2/CEP17* ratio = 1.90, with 27% of cells having a *HER2/CEP17* ratio > 2.2. Both non-amplified (cyan arrows) and amplified (white arrows) nuclei are admixed (original magnification: $\times 1000$, oil immersion).

a single $\times 1000$ oil-immersion field, both *HER2*-non-amplified and (non-clustered) *HER2*-amplified cells can easily be identified, suggestive of a non-uniform population. As seen in the corresponding histogram (Figure 4a), scattered cells with a *HER2/CEP17* ratio of two- or threefold the mean ratio were present within this tumor. The Q–Q plot shows a clear deviation from the diagonal line, by both visual inspection and linear regression analysis (Figure 4b).

Although these two examples illustrate tumors with different distributions, both would be scored as *HER2*-equivocal (ratio of 2.14 in the uniform tumor and 1.90 in the non-uniform tumor). Both tumors would also be classified as 'genetically heterogeneous' (the percentage of amplified cells was 42 and 27%, respectively.)

The Percentage of *HER2*-Amplified Tumor Cells Is Not Informative of the Underlying Distribution

The correlation coefficient (R^2) obtained by linear regression analysis of the Q–Q plot allows the comparison of tumor-cell distribution across different cases. For all 2522 cases, R^2 of the Q–Q plot was plotted against the percentage of amplified cells, to determine whether 'genetically heterogeneous' tumors correspond to genetically non-uniform tumors (Figure 5). There was no correlation between the R^2 value and the percentage of amplified cells.

Discussion

A review of 2522 consecutive cases tested by FISH shows that 'genetic heterogeneity' as defined by the 2009 CAP/ASCO guideline¹⁶ occurs in a significant minority (26%) of all cases, and is not informative of non-uniformity within a tumor-cell population. Rather, the degree of 'genetic heterogeneity' increases as the overall *HER2/CEP17* ratio increases to approach the 'cut point' of 2.2—as statistically expected for any continuous variable. Therefore, 'genetic heterogeneity' does not appear to contribute information about *HER2* amplification independent of the *HER2/CEP17* ratio itself.

The purpose of the 2009 'genetic heterogeneity' definition was to address the possibility that *HER2*-amplified subpopulations of tumor cells may be significant for prognostic and predictive purposes.¹⁶ The 2009 CAP/ASCO guideline was based on expert opinion rather than clinical/pathologic evidence, and has generated some discussion.^{17–19} Nevertheless, the need to collect data on potential *HER2*-amplified subpopulations was cited as a key factor in implementing this guideline.¹⁹ A large European audit performed by Bartlett *et al*²⁰ linked to clinical-trial data showed that the percentage of tumor cells with *HER2/CEP17* ratios > 2.2 does not identify cases with poor clinical outcome, and also that the

Figure 4 Example of a tumor with a non-uniform distribution, highlighted by Q-Q analysis. Example of a case (same as Figure 3) with $HER2/CEP17$ ratio = 1.90, with 27% of cells having a $HER2/CEP17$ ratio > 2.2. As seen in the histogram (a), some of the scattered amplified cells may represent a separate population. Quantile-quantile analysis (b) suggests a corresponding deviation from a normal distribution (R^2 of the actual-vs-theoretical plot = 0.84).

Figure 5 The percentage of $HER2$ -amplified tumor cells is not informative of the underlying distribution. The closeness-of-fit to a normal distribution ($R^2 = 1$), estimated by the R^2 values of Q-Q plots, is plotted against the percentage of tumor cells with a $HER2/CEP17$ ratio > 2.2 ($N = 2522$). Cases defined as 'genetically heterogeneous' (between the dotted lines) are highlighted as hollow squares. There is no correlation between 'genetic heterogeneity' and tumor non-uniformity.

prevalence of 'genetic heterogeneity' is relatively high (up to 33%). Although Bartlett *et al*²⁰ found that a $HER2$ -amplified-cell percentage of 30% may be clinically significant, it is unclear whether this significance is independent of the $HER2/CEP17$ ratio. The current study would suggest the contrary: that this apparent significance of a higher amplified-cell percentage is a statistical artifact. The data therefore suggest that the 2009 'genetic heterogeneity' definition needs reassessment and modification.

Tumors tested by FISH may demonstrate a highly uniform population or one that is widely distributed with respect to $HER2/CEP17$ ratio. Neither the overall ratio nor the percentage of amplified cells

can distinguish between these scenarios. It has been proposed that the presence of scattered amplified cells is an artifact of the technical and biologic aspects of using *in situ* hybridization in the detection of gene amplification.^{14,20} These include variability in cell sampling, completeness of nuclei, and intrinsic variability in tumor aneuploidy.

The 2009 ASCO/CAP guidelines recommend distinguishing between tumors with amplified clusters of cells from those with an admixture of cells.¹⁶ The current study does not address the presence of $HER2$ -amplified cell clusters—it seems reasonable to strive for detection of discrete populations of amplified cells. Notwithstanding this recommendation, two problems remain: (1) how to identify tumors with truly heterogeneous $HER2$ amplification and (2) finding convincing evidence that this heterogeneity is of independent biologic significance beyond simple variability and artifact.

One proposal from Bartlett *et al*²⁰ is to examine the average $HER2/CEP17$ ratio within distinct tumor areas (20–60 nuclei per area), and to define heterogeneous amplification as tumors containing both amplified and non-amplified areas. This approach, although correcting for artifacts detected in individual cells, does not take into account the statistical artifact inherent to defining a numerical threshold. That is, for any continuous variable, individual tumor cells will fall on both sides of any defined threshold. Similarly, one would expect individual tumor areas also to fall on both sides of this threshold. The current analysis shows that this effect is mathematically unrelated to intratumoral heterogeneity, but rather is predictably more pronounced as the average ratio approaches the threshold.

A truly rigorous definition of 'heterogeneity' should take into account the statistical distribution of tumor cells with respect to $HER2/CEP17$ ratio, which requires thorough sampling and counting. Such an approach is difficult and labor intensive. As automated methods of interpreting *in situ*

hybridization based on image analysis become more widely adopted,^{21–23} it may become more feasible to determine the prevalence and significance of true tumor heterogeneity in the future. In the meantime, it must be acknowledged that the *HER2/CEP17* ratio is a continuous variable, and that the definition of 'HER2 amplification' is, by necessity, dichotomous. There remains a lack of data addressing the effectiveness of treatment for tumors that do not fit well into the 'positive' or 'negative' categories.^{3,4,8}

In conclusion, definitions of 'genetic heterogeneity' in *HER2* FISH testing that depend only on the percentage/proportion of amplified cells are not informative of heterogeneity within a tumor population. Rather, the percentage of amplified cells varies with the *HER2/CEP17* ratio in a predictable statistical manner. Current definitions of 'genetic heterogeneity' should be reassessed.

Disclosure/conflict of interest

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