The Dilemma of HER2 Double-equivocal Breast Carcinomas

Genomic Profiling and Implications for Treatment

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Abstract: The American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) 2013 guidelines for HER2 assessment have increased the number of HER2 equivocal breast carcinomas following in situ hybridization reflex testing, that is, HER2 “double equivocal” (equivocal protein expression and equivocal gene copy number). Forty-five double-equivocal carcinomas were subjected to Prosigna analysis. Twenty-seven cases were investigated for the expression of genes found to be differentially expressed between estrogen receptor (ER)-positive/HER2-positive (N = 22) and ER-positive/HER2-negative (N = 22) control cases. Twenty-nine of the 45 cases were also analyzed by targeted sequencing using a panel of 14 genes. We then explored the pathologic complete response rates in an independent series of double-equivocal carcinoma patients treated with trastuzumab-containing chemotherapy. All cases were ER-positive, with a mean Ki67 of 28%. Double-equivocal carcinomas were predominantly luminal B (76%); 9 cases (20%) were luminal A, and 2 cases (4%) HER2-enriched. The majority (73%) showed a high risk of recurrence by Prosigna, even when the carcinomas were small (<2 cm), node-negative/micrometastatic, and/or grade 2. Double-equivocal carcinomas showed TP53 (6/29, 20%), PIK3CA (3/29, 10%), HER2 (1/29, 3%), and MAP2K4 (1/29, 3%) mutations. Compared with grade-matched ER-positive/HER2-negative breast carcinomas from METABRIC, double-equivocal carcinomas harbored more frequently TP53 mutations and less frequently...
PIK3CA mutations (P < 0.05). No significant differences were observed with grade-matched ER-positive/HER2-positive carcinomas. Lower pathologic complete response rates were observed in double-equivocal compared with HER2-positive patients (10% vs. 60%, P = 0.009). Double-equivocal carcinomas are preferentially luminal B and show a high risk of recurrence. A subset of these tumors can be labeled as HER2-enriched by transcriptomic analysis. HER2 mutations can be identified in HER2 double-equivocal cases.

Key Words: breast carcinoma, HER2, equivocal result, molecular subtype, risk of recurrence, mutations

Her2 status assessment is a key step to personalized treatment of breast carcinoma patients, of whom ∼15% are deemed HER2-positive and may benefit from anti-HER2 drugs. The 2013 American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) recommendations for HER2 testing in breast carcinomas led to important changes, not least the adoption of a single cut-off for both immunohistochemistry (IHC) and in situ hybridization (ISH) (> 10%) and the implementation of a second look to assess HER2 copy numbers when dealing with HER2/CEP17 ratios < 2 (ISH algorithm). Moreover, the guidelines provided a more detailed definition of intratumor HER2 genetic heterogeneity, acknowledging the presence of 3 patterns: (i) discrete populations of amplified and non-amplified cells, (ii) diffuse intermingling of amplified and nonamplified cells, (iii) scattered/isolated amplified cells in a predominantly nonamplified tumor. Experts contended that the first pattern represented the only significant type of heterogenous amplification, as interobserver reproducibility is more significant, and data on the clinical impact of intermingled or scattered cell heterogeneity are lacking.

We and others have shown that the 2013 ASCO/CAP guidelines led to an increase in the number of score 2+ cases with subsequent equivocal results. If ISH reflex tests are not effective, these cases are labelled as “double-equivocal” (equivocal HER2 protein expression and equivocal HER2 copy number). Some have suggested that the use of alternative genetic heterogeneity, acknowledging the presence of 3 patterns: (i) discrete populations of amplified and non-amplified cells, (ii) diffuse intermingling of amplified and nonamplified cells, (iii) scattered/isolated amplified cells in a predominantly nonamplified tumor. Experts contended that the first pattern represented the only significant type of heterogenous amplification, as interobserver reproducibility is more significant, and data on the clinical impact of intermingled or scattered cell heterogeneity are lacking.

We and others have shown that the 2013 ASCO/CAP guidelines led to an increase in the number of score 2+ cases with subsequent equivocal results. If ISH reflex tests are not effective, these cases are labelled as “double-equivocal” (equivocal HER2 protein expression and equivocal HER2 copy number). Some have suggested that the use of alternative genetic heterogeneity, acknowledging the presence of 3 patterns: (i) discrete populations of amplified and non-amplified cells, (ii) diffuse intermingling of amplified and nonamplified cells, (iii) scattered/isolated amplified cells in a predominantly nonamplified tumor. Experts contended that the first pattern represented the only significant type of heterogenous amplification, as interobserver reproducibility is more significant, and data on the clinical impact of intermingled or scattered cell heterogeneity are lacking.

Therefore, we sought to stratify double-equivocal carcinomas by using transcriptomics, which allowed for the assessment of RNA expression changes of this specific tumor cell population; on the basis of the transcriptomic heterogeneity of clinically HER2-positive and HER2-negative carcinomas and by the recent demonstration that the identification of HER2-enriched subtype has been associated with better response to anti-HER2 treatment within HER2-positive carcinomas we were particularly intrigued by the molecular subgroup distribution across HER2 double-equivocal carcinomas. Moreover, we investigated the prevalence of somatic mutations affecting the genes most frequently mutated in breast carcinomas, including HER2. Finally, as a hypothesis-generating study we explored the response rate of double-equivocal carcinoma patients treated with trastuzumab-containing chemotherapy.

Materials and Methods

Cohort and Fluorescence ISH

Forty-five breast carcinomas scored as 2+ by IHC and harboring a HER2/CEP17 ratio < 2 and HER2 gene copy numbers ≥ 4 and < 6 by fluorescence in situ hybridization (FISH) were collected from the Pathology Division, Azienda Ospedaliera Città della Salute e della Scienza di Torino/University of Turin (N = 29) and the Pathology Division, European Institute of Oncology, Milan (N = 16). As per both laboratory protocols, sections for FISH had been cut at 4 µm. In addition to the original FISH scoring, 4 µm thick sections were cut to retest FISH on the entire cohort, as previously described. Scoring was performed by 2 independent observers (A.S./C.M.) with expertise in HER2 FISH analysis, who recorded mean HER2 and CEP17 copy numbers, HER2/CEP17 ratios, and prevalence and type of heterogeneity. Consensus was reached on the different patterns of heterogeneity described in the guidelines. Whenever HER2 heterogeneity was detected, FISH results were reported either as whole (mean of HER2 and CEP17 copy numbers of both amplified and nonamplified cells) or separate populations (mean HER2 and CEP17 copy numbers and HER2/CEP17 ratios calculated within distinct populations).

Prosigna Assay

Representative formalin-fixed paraffin-embedded blocks of the 45 cases were sectioned to run Prosiga (NanoString Technologies, Seattle, WA) following the manufacturer’s instructions and as previously reported (Supplementary Methods, Supplemental Digital Content 1, http://links.lww.com/PAS/A652).

Gene expression measurements were converted into intrinsic molecular subtypes, risk of recurrence (ROR) scores, and risk categories using a fully prespecified algorithm. Briefly, the Prosiga Breast Cancer Prognostic Gene Signature Assay Reporter CodeSet and Capture ProbeSet reagents contain a library of probes targeting the 50-gene sequences comprising PAM50. In addition, a set of probes targeting 8 housekeeper genes are included as a normalization tool for the assay. The assay algorithm...
enables a continuous ROR score, which is currently referred to as the Prosinga score in the assay report. The Prosinga score is calculated by multiplying the Pearson correlation to a 46-gene subset of the 50 genes used to calculate 4 molecular subtypes (Luminal A, Luminal B, HER2-enriched, Basal-like), a proliferation score (mean expression of an 18-gene subset of the 50 genes), and tumor size by specific coefficients. The coefficients were learned with a multivariate Cox proportional hazards model using measured values for each test variable from formalin-fixed paraffin-embedded breast cancer samples. The weighted test variables are then summed to produce the Prosinga score. The Prosinga score is reported as an integer on a 1 to 100 scale. A Pearson correlation to a 46-gene subset of the 50 genes is used to determine a value for 4 molecular subtypes. The Prosinga score (range: 0 to 100) is calculated as follows: Prosinga score = 54.769 × (-0.0067 × Basal-like Pearson correlation + 0.4317 × HER2-enriched Pearson correlation - 0.3172 × Luminal A Pearson correlation + 0.4894 × Luminal B Pearson correlation + 0.1981 × Proliferation score + 0.1133 × Tumor size) + 0.8826.

In a previous testing with the PAM50, the ROR score provided a continuous estimate of the ROR for estrogen receptor (ER)-positive, node-negative patients who were treated with tamoxifen for 5 years.15,16

Global Transcriptomics by Microarray Analysis and Validation by NanoString

Two control groups of ER-positive carcinomas, N = 22 HER2-negative (IHC score 0/HER2-non-amplified) and N = 22 HER2-positive (HER2 IHC score 3+/HER2-amplified), were subjected to global transcriptomics by Whole-Genome DASL assay (Illumina Inc., San Diego, CA) according to the manufacturer’s instructions (Supplementary Methods and related Supplementary Figures, Supplemental Digital Content 1, http://links.lww.com/PAS/A652). Genes with differential expression in HER2-positive versus HER2-negative carcinomas were identified on the basis of t test significance P < 0.01 and on mean gene expression variations > ± 2-fold. Cluster analysis was performed using GEDAS software and the “Fuzzy Self-organizing Maps” algorithm with cosenic distance.17

Subsequently, these 2 cohorts and the double-equivocal carcinomas confirmed at least by 2 FISH observers were analyzed by a customized nCounter GX CodeSet assay (NanoString) including the gene signature obtained by DASL, 4 housekeeping genes, 6 positive quality controls, and 8 negative quality controls (Supplementary Methods, Supplemental Digital Content 1, http://links.lww.com/PAS/A652). Analysis of genes significantly differentially expressed between subgroups was performed in MeV 4.8 software (version 10.2) using the t test (critical P-value = 0.05). Unsupervised clustering was performed by nSolver 3.0 (NanoString).

Mutational Analysis by Targeted Sequencing and Comparison With the METABRIC Data Set

Twenty-nine of the 45 cases were investigated for the presence of 140 mutations by a 14-gene breast cancer panel using the MassARRAY System (Agena Bioscience, Hamburg, Germany) (Supplementary Methods, Supplemental Digital Content 1, http://links.lww.com/PAS/A652. Supplementary Table 1, Supplemental Digital Content 2, http://links.lww.com/PAS/A653). The mutational frequencies of the HER2 double-equivocal carcinomas were compared with cases in the METABRIC cohort.18,19 METABRIC cases were ER, histologic subtype, histologic grade (G), and PAM50 matched to HER2 double-equivocal cases at a 10:1 ratio. Additional comparisons involved ER-positive/HER2-negative METABRIC cases (G and subtype matched to HER2 double-equivocal carcinomas at a 10:1 ratio) and ER-positive/HER2-positive METABRIC cases (G and subtype matched to HER2 double-equivocal carcinomas at a 2:1 ratio, Supplementary Methods, Supplemental Digital Content 1, http://links.lww.com/PAS/A652). Somatic mutations in PIK3CA, TP53, ERBB2, and MAP2K4 were extracted, and only hotspot mutations included in our panel were taken into account (Supplementary Methods, Supplemental Digital Content 1, http://links.lww.com/PAS/A652). Comparisons were performed using Fisher exact tests. P-values < 0.05 were considered statistically significant.

Breast Carcinomas Treated With Neoadjuvant Anti-HER2 Therapy

We collected pathologic response data20 of an independent series of 40 breast carcinoma patients who received a sequence of anthracycline-based therapy followed by a taxane with concomitant trastuzumab for a total duration of 24 weeks in the neoadjuvant setting. This cohort comprised 10 HER2 double-equivocal invasive carcinomas of no special type (IC-NSTs) that were matched 1:3 with IC-NSTs showing HER2 overexpression (score 3+ by IHC) and HER2 amplification (n = 30). The 2 subgroups displayed comparable ER status and Ki67 indices (Supplementary Table 2, Supplemental Digital Content 3, http://links.lww.com/PAS/A654). The ten double-equivocal carcinomas showed a non-negligible degree (range: 11% to 44%; mean: 19%) of tumor cells harboring ≥ 6 HER2 copies (range: 6.4 to 8; mean: 7.1) that could be interpreted as HER2 genetic heterogeneity in the form of diffuse intermingling of amplified and nonamplified cells.2

As a negative control group of patients treated with chemo-therapy alone, we referred to a series of neoadjuvant treated patients we previously reported.21 A cohort of 152 ER-positive/HER2-negative patients with Ki67 indices comparable to double-equivocal carcinomas was extracted (Supplementary Table 3, Supplemental Digital Content 4, http://links.lww.com/PAS/A655).

RESULTS

Clinicopathologic Features of Double-equivocal Carcinomas

Complete clinicopathologic details of the cohort are reported in Table 1. Most cases were IC-NSTs (39/45, 87%) and G2 (58%, 26/45). All cases were ER-positive with over 50% positive cells, and 62% (28/45) showed progesterone
| N | Code   | ER (%) | PR (%) | Ki67 (%) | Probability of Recurrence | Mean HER2 c.n. | Mean CEP17 c.n. | HER2/ CEP17 % HT | Histologic Type | Identified Mutations (VAF) | Molecular Subgroup |
|---|--------|--------|--------|----------|---------------------------|---------------|----------------|-----------------|----------------|----------------------------|------------------|
|   | EQV    | 98     | 98     | 7        | Low                       | 18            | 4              | 3.4             | 1.1            | 2                         | Luminal A         |
| 2 | EQV    | 96     | 0      | 10       | High                      | 48            | 4              | 2.4             | 1.7            | <1 Mixed IC-NST/ILC       | Luminal A         |
| 3 | EQV    | 100    | 30     | 27       | Intermediate              | 55            | 4              | 3.8             | 1.1            | <1 Mixed IC-NST           | NA               |
| 4 | EQV    | 99     | 90     | 35       | Intermediate              | 57            | 4              | 3.3             | 1.2            | 20 Mixed IC-NST           | NA               |
| 5 | EQV    | 100    | <1     | 28       | Intermediate              | 55            | 4              | 2.7             | 1.5            | 13 Mixed IC-NST           | NA               |
| 6 | EQV    | 99     | 15     | 25       | High                      | 73            | 4              | 3.2             | 1.2            | 12 Mixed IC-NST           | Luminal B         |
| 7 | EQV    | 98     | 55     | 21       | High                      | 75            | 5              | 2.9             | 1.7            | 3 Mixed IC-NST            | Luminal B         |
| 8 | EQV    | 98     | 98     | 31       | High                      | 65            | 5              | 2.6             | 1.9            | 2 Mixed IC-NST            | Luminal B         |
| 9 | EQV    | 95     | 40     | 25       | High                      | 72            | 4.02           | 2.45            | 1.64           | 5 IC-NST                | Luminal B         |
| 10| EQV    | 95     | 0      | 23       | Intermediate              | 46            | 4.03           | 2.13            | 1.89           | 2 IC-NST                | Luminal B         |
| 11| EQV    | 95     | 5      | 31       | High                      | 84            | 4.08           | 2.48            | 1.64           | 5 IC-NST                | Luminal B         |
| 12| EQV    | 98     | 20     | 26       | High                      | 71            | 4.1            | 3.2             | 1.3            | 4 IC-NST                | Luminal B         |
| 13| EQV    | 98     | 45     | 24       | High                      | 76            | 4.1            | 2.5             | 1.7            | 3 IC-NST                | Luminal B         |
| 14| EQV    | 99     | 60     | 17       | High                      | 62            | 4.1            | 3.5             | 1.1            | 1 IC-NST                | Luminal B         |
| 15| EQV    | 95     | 10     | 22       | High                      | 62            | 4.1            | 3.9             | 1.3            | 1 IC-NST                | Luminal B         |
| 16| EQV    | 95     | 75     | 60       | High                      | 82            | 4.1            | 3.9             | 1.5            | 5 IC-NST                | Luminal B         |
| 17| EQV    | 99     | 10     | 36       | High                      | 86            | 4.1            | 2.8             | 1.5            | 12 IC-NST               | Luminal B         |
| 18| EQV    | 98     | 50     | 15       | High                      | 69            | 4.2            | 3.7             | 1.1            | 3 IC-NST                | Luminal B         |
| 19| EQV    | 95     | 10     | 20       | High                      | 71            | 4.2            | 3.1             | 1.3            | 3 IC-NST                | Luminal B         |
| 20| EQV    | 95     | 5      | 40       | High                      | 62            | 4.2            | 3.8             | 1.1            | 3 IC-NST                | Luminal B         |
| 21| EQV    | 98     | 3      | 36       | High                      | 89            | 4.2            | 3.4             | 1.2            | 5 IC-NST                | Luminal B         |
| 22| EQV    | 95     | 80     | 25       | Intermediate              | 53            | 4.2            | 2.33            | 1.8            | 5 IC-NST                | Luminal B         |
| 23| EQV    | 98     | 90     | 16       | Low                       | 30            | 4.2            | 3.9             | 1.1            | <1 Mixed IC-NST           | Luminal B         |
| 24| EQV    | 90     | 50     | 15       | Low                       | 49            | 4.2            | 2.8             | 1.5            | 15 IC-NST               | Luminal B         |
| 25| EQV    | 95     | 60     | 25       | Low                       | 99            | 4.3            | 3.4             | 1.3            | 5 IC-NST                | Luminal B         |
| 26| EQV    | 90     | 70     | 40       | Low                       | 66            | 4.3            | 3.6             | 1.2            | <1 Mixed IC-NST           | Luminal B         |
| 27| EQV    | 99     | 15     | 5        | Low                       | 4             | 4.3            | 2.8             | 1.5            | 15 IC-NST               | Luminal A         |
| 28| EQV    | 95     | 70     | 20       | Intermediate              | 19            | 4.43           | 2.37            | 1.87           | 10 Mixed IC-NST          | Luminal A         |
| 29| EQV    | 95     | 95     | 28       | High                      | 81            | 4.46           | 2.25            | 1.95           | 3 Mixed IC-NST           | Luminal B         |
| 30| EQV    | 90     | 90     | 35       | High                      | 81            | 4.47           | 2.6             | 1.72           | 18 Mixed IC-NST          | Luminal B         |
| 31| EQV    | 95     | 90     | 26       | High                      | 78            | 4.5            | 4.1             | 1.1            | 3 IC-NST                | Luminal B         |
| 32| EQV    | 100    | 15     | 30       | High                      | 81            | 4.5            | 3.4             | 1.3            | 25 IC-NST               | Luminal B         |

**Table 1.** Cohort of 45 Breast Carcinomas Harboring a Double-equivocal Result for the HER2 Status
receptor expression in over 20% of tumor cells. Proliferation indices ranged between 5% and 60% (mean: 28%); 38% of cases (17/45) had proliferation indices ≥30%, and the large majority (34/45, 75%) showed a Ki67 above the 20% threshold. A low proliferation index (<10%) was occasionally found (cases 1, 27, and 40). Of note, equivocal carcinomas showed a high ROR (mean value: 49.3; range: 45 to 55; mean value of high RORs: 71.2; range of high RORs: 45-99), even when considering only unifocal small (<2 cm) node-negative/micrometastatic carcinomas (13/23, 56%; mean: 73%, median: 71.2%); ROR range: 4 to 86; mean value of high RORs: 73.3; range of high RORs: 45-99), and 2 (4%) were HER2-enriched.

### FISH Patterns

In 27 of 45 cases (60%), all observers agreed on the presence of a homogeneously HER2-equivocal tumor cell population (4 ≤ HER2 copy number > 6) (Figs. 1, 2). In 12 additional cases (27%), the equivocal range was confirmed by all observers who also identified aggregated tumor cells accounting for >10% of the tumor population (range: 12% to 30%; mean: 20%) harboring ≥6 HER2 signals (Supplementary Fig. 1, Supplemental Digital Content 5, http://links.lww.com/PAS/A656, Supplementary Table 4, Supplemental Digital Content 6, http://links.lww.com/PAS/A657). Finally, six cases (13%) were scored as HER2-negative by 2/3 observers.

### Molecular Subtyping

The large majority (34/45, 76%) of cases were classified as luminal B, 9 (20%) fell in the luminal A subgroup, and 2 (4%) were HER2-enriched.

Double-equivocal carcinomas frequently (33/45, 73%) showed a high ROR (mean: 64; range: 4 to 99; mean value of high RORs: 73.3; range of high RORs: 45-99), even when considering only unifocal small (<2 cm) node-negative/micrometastatic carcinomas (13/23, 56%; mean: 77; range: 4-86; mean value of high RORs: 71.2; range of high RORs: 56-86) (Table 1 and Fig. 2). Within the 17 G2 carcinomas with a tumor size <2 cm and which were node-negative/micrometastatic (13/23, 56%; mean: 73; range: 4-86; mean value of high RORs: 71.2; range of high RORs: 56-86) (Table 1 and Fig. 2). Within the 17 G2 carcinomas with a tumor size <2 cm and which were node-negative/micrometastatic, 9 (53%), 4 (23.5%), and 4 (23.5%) showed high, intermediate, and low ROR, respectively (ROR mean: 63.5; ROR range: 4 to 86; Table 1). Within the 9 luminal A carcinomas, 3 (33%) showed a high ROR (mean value: 49.3; range: 45 to 55; Table 1).

### HER2 mRNA Levels

HER2 mRNA levels extrapolated by the NanoString custom assay were significantly different between HER2-positive, HER2-negative, and HER2 double-equivocal carcinomas (P <0.0001, ANOVA test, Fig. 3A and B). A greater overlap in terms of HER2 mRNA levels was observed between HER2-negative and HER2-equivocal carcinomas than between HER2-equivocal and HER2-positive carcinomas.
Transcriptomic Stratification

A list of 24 genes differentially expressed between HER2-positive/ER-positive and HER2-negative/ER-positive tumors was derived from global transcriptomics and further investigated using the NanoString custom assay. Fourteen genes were confirmed as differentially expressed between the 2 groups and investigated in the subset of 27 double-equivocal carcinomas showing a homogenous population of cells with HER2 copy number in the equivocal range (Supplementary Table 5, Supplemental Digital Content 7, http://links.lww.com/FIGURE 1. HER2 expression and HER2/CEP17 pattern by FISH in double-equivocal carcinomas. A and B represent examples of a double-equivocal breast carcinoma pertaining to the luminal B subgroup by Prosigna. C and D illustrate one of the 2 cases that were labeled as HER2-enriched by Prosigna; this case was also found to harbor a subclonal L755 HER2 truncating mutation affecting the kinase domain. E and F depict one of the 2 double-equivocal carcinomas that clustered together with HER2-positive carcinomas on the basis of the 14-gene signature and that was luminal B by Prosigna.
This 14-gene signature included HER2 and genes pertaining to the HER2 amplicon, together with genes (AGTR1, NOVAl, TPRGl) whose expression appeared as mutually exclusive with HER2, when analyzed in the Cancer Genome Atlas (TCGA) breast cancer data set (www.cbioportal.org, study: Breast Invasive Carcinoma—TCGA provisional; n = 1105) (Supplementary Fig. 2, Supplemental Digital Content 8, http://links.lww.com/PAS/A659).

Unsupervised clustering produced 2 main clusters, which differed by the expression of HER2 amplicon-related genes (Fig. 3). The cluster showing lower levels of these genes comprised one subcluster characterized by heterogenous and intermediate expression levels of HER2 amplicon-related genes, and another subcluster with cases showing low expression levels of HER2 amplicon-related genes as well as HER2 anticorrelated genes. The large majority (25/27) of HER2-equivocal carcinomas clustered with HER2-negative carcinomas, whereas all but one HER2-positive carcinoma grouped within the cluster enriched for HER2 amplicon-related genes, together with 2 double-equivocal carcinomas classified as luminal B (Fig. 3; P < 0.0001, χ² test).

**Mutations in Cancer Genes**

Recurrent mutations were found in TP53 (6/29, 20%) and PIK3CA (3/29, 10%); one case showed a MAP2K4 mutation (3%) and another case (3%), “HER2-enriched” by Prosigna, harbored a subclonal (variant allele frequency = 11%) truncating L755×HER2 mutation (Table 1).

Double-equivocal carcinomas showed a significantly higher frequency of TP53 mutations and a significantly lower frequency of PIK3CA mutations (P = 0.007 and 0.018, respectively, Table 2) compared with that of ER-positive, grade-matched and molecular subtype–matched cohort of cases from the METABRIC data set. This held true when compared with grade-matched ER-positive/HER2-negative cases (P = 0.003 and 0.001, respectively), whereas no significant differences were observed with grade-matched ER-positive/HER2-positive carcinomas (Table 2).

**Pathologic Complete Response Rates in HER2 Double-equivocal Carcinomas Treated With Trastuzumab-containing Chemotherapy**

The pathologic complete response (pCR) rate within the cohort of double-equivocal carcinomas was significantly lower than in ER-matched and Ki67-matched HER2 score 3+ carcinomas (10% vs. 60%, Fisher exact test, P = 0.009, Supplementary Table 6, Supplemental Digital Content 9, http://links.lww.com/PAS/A660). Three cases showed a near pCR (minimal residual disease/near total effect/< 10% of tumor remaining). When pCR and near-pCR categories were grouped, the difference in terms
FIGURE 3. Gene expression analysis. A and B, HER2 mRNA levels in double-equivocal breast carcinomas in comparison with HER2-positive and HER2-negative carcinomas. HER2 mRNA levels (y-axis, extrapolated from the NanoString custom assay) were significantly different between HER2-positive (score 3+), HER2-negative (score 0), and HER2 double-equivocal carcinomas (x-axis) (P < 0.0001, ANOVA test). D-Equiv: HER2 double-equivocal carcinomas. In A, the double-equivocal carcinomas are represented altogether in a single group. Comparison between HER2-equivocal versus HER2-positive and HER2-equivocal versus HER2-negative were both statistically significantly different (t test, P < 0.0001 and 0.0048, respectively). In B, double-equivocal carcinomas are subgrouped into those confirmed by 2/3 observers (EEN) and those confirmed by 3/3 observers (EEE); in addition, we separated score 0 cases into those in which no staining was observed and those wherein incomplete and faint/barely perceptible membrane staining within ≤10% of tumor cells could be appreciated. HER2 mRNA levels of these subgroups were compared with those of HER2-equivocal: the difference between HER2-equivocal versus score 0-negative carcinomas was significant (t test, P = 0.014), whereas the difference between HER2-equivocal versus score 0 to <10% did not reach statistical significance (t test, P = 0.066). C, Hierarchical clustering of ER-positive/HER2-double-equivocal, ER-positive/HER2-positive, and ER-positive/HER2-negative breast carcinomas based on a gene signature of 14 genes found to be differentially expressed between the 2 cohorts of ER-positive/HER2-positive and ER-positive/HER2-negative carcinomas. Cases are represented in columns; genes are depicted in rows. All HER2-positive carcinomas except one grouped within the cluster enriched for HER2 amplicon-related genes together with 2 double-equivocal carcinomas classified as luminal B by Prosigna (black asterisks); the large majority of HER2-equivocal carcinomas preferentially clustered with HER2-negative carcinomas and one HER2-positive carcinoma (red asterisk) in the remaining 2 clusters (P < 0.0001, \( \chi^2 \) test). The 2 Prosigna HER2-enriched carcinomas (blue asterisks) clustered with those characterized by nonhomogenous expression of HER2 amplicon-related genes as well as HER2 anticorrelated genes. The control cohorts comprised 19 of the original 22 cases, as 3 cases in each subset showed low Pearson correlation coefficients, when compared with the rest of the cases within each cohort. CTRL 0: breast carcinoma of the control group of HER2-negative cases (score 0); CTRL 3: breast carcinoma of the control group of HER2-positive cases (score 3+).
of response rate was not statistically significant between double-equivocal and score 3+ carcinomas (40% vs. 63.3%, \( P = 0.27 \), Fisher exact test, Supplementary Table 7, Supplemental Digital Content 10, http://links.lww.com/PAS/A661).

Notably, the response rates in a series of 152 ER-positive/HER2-negative carcinomas were numerically lower (27%) compared with that observed in double-equivocal carcinomas that received trastuzumab in addition to chemotherapy (40%; Fisher exact test, \( P = 0.46 \), Supplementary Table 8, Supplemental Digital Content 11, http://links.lww.com/PAS/A662).

**DISCUSSION**

Here we show that double-equivocal carcinomas represent a rather heterogenous group of breast carcinomas that are preferentially luminal B at the transcriptomic level and whose HER2 mRNA levels show a greater overlap with HER2-negative rather than with HER2-positive carcinomas. Nevertheless, TP53 and PIK3CA mutation rates are more similar to those found in ER-positive/HER2-positive carcinomas than those observed in ER-positive/HER2-negative carcinomas, and a subgroup of double-equivocal cases can be defined “HER2-enriched” at the transcriptomic level and may harbor pathogenic HER2 mutations.

In line with recent reports,\(^4\) we observed subclonal tumor populations with a variable range of HER2 copy numbers (HER2 genetic heterogeneity) in double-equivocal carcinomas. The prognostic and predictive relevance of subclonal populations of cells with a copy number \( \geq 6 \) within otherwise nonamplified tumors remains a topic of debate. Of note, in our series, these cases typically showed relatively low HER2 copy numbers and mostly harbored HER2/CEP17 ratios \(< 2 \) because of co-occurrence of CEP17 gains, as also observed by others.\(^4,22\) These features may suggest complex rearrangements in chromosome 17 that merit further investigation. From a clinical standpoint, the key question is whether these genetically heterogenous tumors respond to trastuzumab. We explored this question in a cohort of patients treated with neoadjuvant trastuzumab-containing chemotherapy and observed that pCR rates were significantly lower in double-equivocal carcinomas with HER2 heterogeneity compared with score 3+ carcinomas. Although our analysis is limited by a small number of cases, these results are in line with recent data.\(^23,24\) When pCR and near-pCR categories were grouped together, the response rates in double-equivocal carcinomas reached 40%; however, we cannot rule out that this was due to the beneficial effect of chemotherapy, as this rate was not significantly different from the rates accrued in a cohort of ER-positive/HER2-negative patients treated with chemotherapy only. It should be emphasized that this is a hypothesis-generating study and that larger studies comparing patients who received the same chemotherapy regimens +/- trastuzumab treatment are warranted to ascertain the real impact of anti-HER2 therapy in this specific subset of breast carcinomas.

As well as containing carcinomas with some degree of HER2 genetic heterogeneity, our series was substantially composed of tumors featuring homogeneously HER2-equivocal tumor cell populations. Whether the HER2 gain/HER2 protein expression showed by these tumor cells is capable of driving significant HER2 pathway activation that may be sensitive to anti-HER2 agents is unknown and difficult to assess. Currently, only comparative data between double-equivocal and HER2-negative carcinomas treated with chemotherapy regimens are available and suggest comparable outcomes between the 2 categories.\(^25\) Whether patients affected by double-equivocal carcinomas do better with the addition of anti-HER2 agents has yet to be determined. In addition, data on the prognostic impact of double-equivocal HER2 status remain controversial.\(^26,28\) Our results on the relatively high frequency of TP53 mutations may support the contention that these cases constitute an aggressive subgroup of ER-positive carcinomas. This is in line with the risk-based stratification of the cohort provided by Prosigna analysis, which demonstrated high RORs, even within the subgroup of node-negative G2 carcinomas with tumor size \(< 2 \text{ cm} \). The clinical utility of the Prosigna assay in double-equivocal carcinomas may also be relevant because of the molecular subtyping. The possibility to label a breast carcinoma otherwise classified as equivocal as “HER2-enriched” opens up the possibility to explore the beneficial effect of anti-HER2 agents in ad hoc window of
opportunity trials. Interestingly, one of the 2 HER2-enriched tumors harbored a subclonal L755 HER2 truncating mutation. Although likely deleterious, as it is a truncating mutation affecting the HER2 kinase domain, its clinical significance is unknown at present. Nevertheless, the potential identification of HER2 mutations should be considered in HER2 double-equivocal cases, as this may be of clinical relevance, given the recent results on the response rates to neratinib in breast carcinoma patients harboring oncogenic HER2 mutations.29

As a further level of complexity, stratification of double-equivocal carcinomas by means of a gene signature derived from ER-positive/HER2-positive and ER-positive/HER2-negative carcinomas showed that double-equivocal carcinomas preferentially clustered with HER2-negative cases, and a subgroup expressed high levels of genes that negatively correlate with HER2 in the TCGA data set. Of these, AGTR1 has been described as a potential therapeutic target for ER-positive/HER2-negative breast carcinomas30,31 and linked to resistance to neoadjuvant chemotherapy in HER2-negative breast carcinomas.32

Our study has several limitations, including the small sample size. Nevertheless, double-equivocal carcinomas are relatively rare, need ISH testing to be recognized, and the cases analyzed here were selected from 2 institutions with high ISH testing workloads per year. Second, the mutational analysis was limited to the hotspots of the most frequently mutated genes in breast cancer. We cannot exclude that whole-exome sequencing may reveal potentially actionable, key driver alterations in this subgroup. Finally, no follow-up information for this cohort was available. Nevertheless, a surrogate of outcome stemmed from the multigene prognostic signature analysis.

Despite these limitations, our thorough genomic characterization of breast carcinomas with double-equivocal HER2 status demonstrates the possibility of biologically and prognostically stratifying these carcinomas, a feature that may be instrumental to support treatment decision-making. A single group assignment study called EQUI-VOK (NCT03197805, ClinicalTrials.gov) is currently investigating the impact of RNA genomic profiling on treatment decision-making in this subgroup of breast carcinoma patients. It is important to note that, if on the one side double-equivocal breast carcinomas are frequently luminal B and preferentially cluster with HER2-negative rather than HER2-positive carcinomas on the basis of their gene expression profiles, on the other side a subset of these tumors are classified as HER2-enriched and harbor a mutation profile more consistent with HER2-positive rather than HER2-negative carcinomas. Our study prompts the need to investigate in clinical trials whether patients with double-equivocal breast carcinomas, including those classified as HER2-enriched, may benefit from anti-HER2 therapies.

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REFERENCES

1. Sapino A, Goia M, Recuppero D, et al. Current challenges for HER2 testing in diagnostic pathology: state of the art and controversial issues. Front Oncol. 2013;3:129.
2. Wolff AC, Hammond ME, Hicks DG, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol. 2015;33:3997–4013.
3. Sapino A, Maletta F, Verdon di Cantogno L, et al. Gene status in HER2 equivocal breast carcinomas: impact of distinct recommendations and contribution of a polymerase chain reaction-based method. Oncologist. 2014;19:1118–1126.
4. Ballard M, Jalikis F, Krings C, et al. “Non-classical” HER2 FISH results in breast cancer: a multi-institutional study. Mod Pathol. 2017;30:227–235.
5. Shah MV, Viktor AE, Meyer RG, et al. Change in pattern of HER2 fluorescent in situ hybridization (FISH) results in breast cancers submitted for FISH testing: experience of a reference laboratory using US Food and Drug Administration Criteria and American Society of Clinical Oncology and College of American Pathologists Guidelines. J Clin Oncol. 2016;34:3502–3510.
6. Ragazzi M, Bisagni A, Gasparini E, et al. Impact of 2013 ASCO/CAP guidelines on HER2 determination of invasive breast cancer: a single institution experience using frontline dual-color FISH. Breast. 2017;34:65–72.
7. Long TH, Lawce H, Durum C, et al. The new equivocal: changes to HER2 FISH results when applying the 2013 ASCO/CAP Guidelines. Am J Clin Pathol. 2015;144:253–262.
8. Marchio C, Lambros MB, Gugliotta P, et al. Does chromosome 17 centromere copy number predict polysomy in breast cancer? A fluorescence in situ hybridization and microarray-based CGH analysis. J Pathol. 2009;219:16–24.
9. Rondon-Lagos M, Verdon Di Cantogno L, Rangel N, et al. Unraveling the chromosome 17 patterns of FISH in interphase nuclei: an in-depth analysis of the HER2 amplicon and chromosome 17 centromere by karyotyping, FISH and M-FISH in breast cancer cells. BMC Cancer. 2014;14:922.
10. Llombart-Cussac A, Cortes J, Pare L, et al. HER2-enriched subtype as a predictor of pathological complete response following trastuzumab and lapatinib without chemotherapy in early-stage HER2-positive breast cancer (PAMELA): an open-label, single-group, multicentre, phase 2 trial. Lancet Oncol. 2017;18:545–554.
11. Perez EA, Ballman KV, Mashadi-Hosseini A, et al. Intrinsic subtype and therapeutic response among HER2-positive Breast Stumors from the NCCCTG (Alliance) N9831 Trial. J Natl Cancer Inst. 2016;109:pii: djw017.
12. Prat A, Carey LA, Adamo B, et al. Molecular features and survival outcomes of the intrinsic subtypes within HER2-positive breast cancer. J Natl Cancer Inst. 2014;106:pii: dju152.
13. Carey LA, Berry DA, Cirrincione CT, et al. Mutations and contribution of a polymerase chain reaction-based method. Oncologist. 2014;19:1118–1126.
14. Walden B, Storhoff J, Nielsen T, et al. Development and verification of the PAM50 risk of recurrence score with oncotype DX and IHC4 for predicting risk of distant recurrence after endocrine therapy. J Clin Oncol. 2013;31:2783–2790.

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16. Gnant M, Filipits M, Greil R, et al. Predicting distant recurrence in receptor-positive breast cancer patients with limited clinicopathological risk: using the PAM50 Risk of Recurrence score in 1478 postmenopausal patients of the ABCSG-8 trial treated with adjuvant endocrine therapy alone. Ann Oncol. 2014;25:339–345.

17. Fu L, Medico E. FLAME, a novel fuzzy clustering method for the analysis of DNA microarray data. BMC Bioinformatics. 2007;8:3.

18. Curtis C, Shah SP, Chin SF, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature. 2012;486:346–352.

19. Pereira B, Chin SF, Rueda OM, et al. The somatic mutation profiles of 2,433 breast cancers refines their genomic and transcriptomic landscapes. Nat Commun. 2016;7:11479.

20. Pinder SE, Provenzano E, Earl H, et al. Laboratory handling and histology reporting of breast specimens from patients who have received neoadjuvant chemotherapy. Histopathology. 2007;50:409–417.

21. Balmativola D, Marchio C, Maule M, et al. Pathological non-response to chemotherapy in a neoadjuvant setting of breast cancer: an inter-institutional study. Breast Cancer Res Treat. 2014;148:511–523.

22. Swanson PE, Yang H. Is “Polysomy” in breast carcinoma the “New Equivocal” in HER2 testing? Am J Clin Pathol. 2015;144:181–184.

23. Landmann A, Farrugia DJ, Diego E, et al. HER2 equivocal breast cancer and neoadjuvant therapy: is response similar to HER2-positive or HER2-negative tumors? J Clin Oncol. 2016;34(suppl):612.

24. Hou Y, Nitta H, Wei L, et al. HER2 intratumoral heterogeneity is independently associated with incomplete response to anti-HER2 neoadjuvant chemotherapy in HER2-positive breast carcinoma. Breast Cancer Res Treat. 2017;166:447–457.

25. Press MF, Sauter G, Buyse M, et al. HER2 gene amplification testing by fluorescent in situ hybridization (FISH): comparison of the ASCO-College of American Pathologists Guidelines With FISH Scores Used for Enrollment in Breast Cancer International Research Group Clinical Trials. J Clin Oncol. 2016;34:3518–3528.

26. Bisierni GB, Engstrom MJ, Bofin AM. HER2 gene copy number and breast cancer-specific survival. Histopathology. 2016;69:871–879.

27. Cricciuillo C, Bagnardi V, Viale G, et al. HER2 equivocal status in early breast cancer is not associated with higher risk of recurrence. Anticancer Res. 2016;36:3537–3540.

28. Sneige N, Hess KR, Multani AS, et al. Prognostic significance of equivocal human epidermal growth factor receptor 2 results and clinical utility of alternative chromosome 17 genes in patients with invasive breast cancer: a cohort study. Cancer. 2017;123:1115–1123.

29. Hyman DM, Piha-Paul SA, Won H, et al. HER kinase inhibition in patients with HER2- and HER3-mutant cancers. Nature. 2018;554:189–194.

30. Ateeq B, Tomlins SA, Chinnaiyan AM. AGTR1 as a therapeutic target in ER-positive and ERBB2-negative breast cancer cases. Cell Cycle. 2009;8:3794–3795.

31. Rhodes DR, Ateeq B, Cao Q, et al. AGTR1 overexpression defines a subset of breast cancer and confers sensitivity to losartan, an AGTR1 antagonist. Proc Natl Acad Sci U S A. 2009;106:10284–10289.

32. de Ronde JJ, Lips EH, Mulder L, et al. SERPINA6, BEX1, AGTR1, SLC26A3, and LAPTM4B are markers of resistance to neoadjuvant chemotherapy in HER2-negative breast cancer. Breast Cancer Res Treat. 2013;137:213–223.