Adjusting Breast Cancer Patient Prognosis with Non-HER2-Gene Patterns on Chromosome 17

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Abstract

Background: HER2 and TOP2A gene status are assessed for diagnostic and research purposes in breast cancer with fluorescence in situ hybridization (FISH). However, FISH probes do not target only the annotated gene, while chromosome 17 (chr17) is among the most unstable chromosomes in breast cancer. Here we asked whether the status of specifically targeted genes on chr17 might help in refining prognosis of early high-risk breast cancer patients.

Methods: Copy numbers (CN) for 14 genes on chr17, 4 of which were within and 10 outside the core HER2 amplicon (HER2- and non-HER2-genes, respectively) were assessed with qPCR in 485 paraffin-embedded tumor tissue samples from breast cancer patients treated with adjuvant chemotherapy in the frame of two randomized phase III trials.

Principal Findings: HER2-genes CN strongly correlated to each other (Spearman’s rho >0.6) and were concordant with FISH HER2 status (Kappa 0.6697 for ERBB2 CN). TOP2A CN were not concordant with TOP2A FISH status (Kappa 0.1154). CN hierarchical clustering revealed distinct patterns of gains, losses and complex alterations in HER2- and non-HER2-genes associated with IHC4 breast cancer subtypes. Upon multivariate analysis, non-HER2-gene gains independently predicted for shorter disease-free survival (DFS) and overall survival (OS) in patients with triple-negative cancer, as compared to luminal and HER2-positive tumors (interaction p = 0.007 for DFS and p = 0.011 for OS). Similarly, non-HER2-gene gains were associated with worse prognosis in patients who had undergone breast-conserving surgery as compared to modified radical mastectomy (p = 0.004 for both DFS and OS). Non-HER2-gene losses were unfavorable prognosticators in patients with 1–3 metastatic nodes, as compared to those with 4 or more nodes (p = 0.017 for DFS and p = 0.001 for OS).

Conclusions: TOP2A FISH and qPCR may not identify the same pathology on chr17q. Non-HER2 chr17 CN patterns may further predict outcome in breast cancer patients with known favorable and unfavorable prognosis.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. Raw data (CopyCaller v2 for gene target per run) including the overview of runs can be found at http://hecog-images.gr/17CN/.

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Introduction

Chromosomal instability (CIN), defined as losses or gains of multiple chromosomal areas [1], represents one aspect of genome instability that underlies all hallmarks of cancer [2]. Within CIN, amplification, is a common event. For routine diagnostic and research purposes employing formalin-fixed paraffin-embedded tissues (FFPE), gene copies are usually evaluated with fluorescence in situ hybridization (FISH) on interphase chromosomes in truncated nuclei. In breast cancer in particular, FISH assays for the assessment of HER2 (ERBB2) gene status on chromosome 17, as compared to those with 4 or more nodes (p = 0.017 for DFS and p = 0.001 for OS).
and HER2 interpretation guidelines [3]. Triple assays, detecting CEN17, centromeric regions (CEN17) are used for diagnostics, under strict interpretation guidelines [3]. Triple assays, detecting CEN17, HER2 and TOP2A, a gene on 17q21, have also received IVF license. However, as shown with methods evaluating larger parts of, or entire chromosomes, an increased number of HER2 gene copies on 17q12 may occur as a single event on an otherwise stable chr17 or it may accompany a broad spectrum of changes on the same chromosome. In addition, chr17 may be unstable without increased HER2 copies [4–8], or chr17 may not be intact [3,9]. The above aspects of chr17 instability may be relevant to breast cancer patient outcome, at various disease stages and treatment settings, but are missed with the currently used FISH assays and their interpretation, as shown with the use of distinct evaluation of CEN17 signals [10] and application of multiple FISH assays for chr17 [11].

Another concern with the currently used bacterial artificial chromosome probes for FISH is that they span large chromosomal areas; for example, 5 Mb for the CEN17, 600 Kb for the HER2 and 500 Kb for the TOP2A probes in the triple assay. Given that the targeted genes are 53 Kb (ERBB2) and 38 Kb (TOP2A), and that the distance between them and their neighboring genes is occasionally short, FISH probes in fact cover a multitude of genes in addition to the annotated one. Further, these probes need to be cut in pieces of maximally 500 bp for efficient labeling and hybridization. Since 10 Kb fluorescent signals can be detected with most modern image analysis systems, the case may be that the probes in fact detect fragments of the targeted regions, which do not necessarily include the annotated genes.

In the present study we used qPCR for the assessment of somatic copy number (CN) alterations in 14 genes on chr17p and chr17q, including ERBB2 and TOP2A. CN alterations were compared to those assessed by classic FISH and were evaluated for their impact on the outcome of patients with operable high-risk breast cancer treated with anthracycline-containing regimens in the pre-trastuzumab era.

Materials and Methods

The study was performed on FFPE tissues from a series of patients with operable high-risk breast cancer who had been treated within the frame of two randomized phase III trials by the Hellenic Cooperative Oncology Group (HeCOG), HE10/97 [12] and HE10/00 [13,14]. Patients had undergone modified radical mastectomy (MRM) or breast-conserving surgery (BCS) and had received adjuvant E-T-CMF except for the control arm in HE10/97 who had received E-CMF only. Patients with HER2-positive tumors had received trastuzumab upon relapse.

Clinical protocols were approved by local regulatory authorities and were also included in the Australian New Zealand Clinical Trials Registry (ANZCTR) and allocated the following Registration Numbers: ACTRN1261100506998 (HE10/97) and ACTRN12609001036202 (HE10/00). The present translational research protocol was approved by the Bioethics Committee of the Aristotle University of Thessaloniki School of Medicine under the general title “Molecular investigation of the predictive and/or prognostic role of important signal transduction pathways in breast cancer” (A7150/18-3-2008). All patients signed a study-specific written informed consent before randomization, which in addition to giving consent for the trial allowed the use of their biological material for future research purposes. Tumors had previously been subtyped with IHC4 and FISH on tissue microarrays (TMA), including two 1.5 mm cores per tumor [15]. Data for TOP2A gene status were also available [16].

For easily distinguishing between parameters assessed with different methods, throughout this text, HER2 refers to the HER2 amplicon, to results obtained by FISH and array-based comparative genomic hybridization (aCGH), to tumor HER2 status and to breast cancer subtypes, while ERBB2 is used for results obtained specifically for sequences of this gene, e.g. with qPCR methods.

Immunohistochemistry (IHC) and triple FISH

IHC protocols for the ER, PgR, HER2 and Ki67 data used in this study have previously been described [15]. Briefly, HER2 was scored in a 4-scale from 0–3, with intense membrane staining in >30% invasive tumor cells classified as positive (3+ staining) [17]. Cut-offs were set for ER and PgR at 1% positive nuclei [18], and for Ki67 at 14% [19]. For the purposes of the present study, ER and PgR simultaneous staining was considered as one parameter (hormone receptor status, HRS). Ki67 was evaluated as a continuous variable (% of positively stained nuclei); the highest score for each TMA core from the same tumor was recorded. For Ki67 at 14% [19]. For the purposes of the present study, ER and PgR simultaneous staining was considered as one parameter (hormone receptor status, HRS). Ki67 was evaluated as a continuous variable (% of positively stained nuclei); the highest score for each TMA core from the same tumor was recorded. FISH was evaluated in twenty tumor nuclei [20]. The HER2 gene was classified as amplified for HER2/CEN17 ratios ≥2.2 [17], or for mean HER2 copy numbers >6 [21]. The TOP2A/CEN17 ratio cut-off for TOP2A amplification was ≥2.0 [22].
Table 1. Demographic and clinicopathological characteristics of patients and tumors from HE10/00 & HE10/97 cohorts.

| Parameter                              | Categories                      | 5 genes* (HE10/00 & HE10/97) | All 14 genes (HE10/00 only) |
|----------------------------------------|---------------------------------|------------------------------|-----------------------------|
| Patients                               | N                               | 485                          | 376                         |
| Age (continuous)                       | Mean (SD)                       | 53.3 (11.4)                  | 54.3 (11.0)                 |
|                                        | Min–Max                         | 22–79                        | 28–77                       |
| Age (categorical)                      | 34–50                           | 173 (35.6%)                  | 127 (33.8%)                 |
|                                        | <34                             | 20 (4.2%)                    | 11 (3%)                     |
|                                        | >50                             | 292 (60.2%)                  | 238 (63.2%)                 |
| Menopausal Status                      | Post                            | 279 (57.6%)                  | 230 (61.2%)                 |
|                                        | Pre                             | 206 (42.4%)                  | 146 (38.8%)                 |
| Study                                  | HE10/00                         | 411 (84.8%)                  | 376 (100%)                  |
|                                        | HE10/97                         | 74 (15.2%)                   |                             |
| Type of surgery                        | MRM                             | 315 (65%)                    | 236 (62.8%)                 |
|                                        | BCS                             | 170 (35%)                    | 140 (37.2%)                 |
| Tumor size                             | >2 cm                           | 350 (72.2%)                  | 266 (70.8%)                 |
|                                        | ≤2 cm                           | 135 (27.8%)                  | 110 (29.2%)                 |
| Histological grade                    | I–II                            | 216 (44.6%)                  | 169 (45%)                   |
|                                        | III-Undifferentiated            | 269 (55.4%)                  | 207 (55%)                   |
| Histological type                      | Mixed                           | 27 (5.6%)                    | 20 (5.4%)                   |
|                                        | Comedo                         | 11 (2.2%)                    | 9 (2.4%)                    |
|                                        | Medullary                      | 10 (2%)                      | 7 (1.8%)                    |
|                                        | Papillary                      | 3 (0.6%)                     |                             |
|                                        | Inflammatory                   | 6 (1.2%)                     | 3 (0.8%)                    |
|                                        | Invasive ductal                 | 389 (80.2%)                  | 306 (81.4%)                 |
|                                        | Invasive lobular                | 39 (8%)                      | 31 (8.2%)                   |
| N of positive nodes                   | 0                               | 1 (0.2%)                     |                             |
|                                        | 1 to 3                          | 274 (56.4%)                  | 176 (46.8%)                 |
|                                        | >3                              | 210 (43.2%)                  | 200 (53.2%)                 |
| Treatment**                            | E-T-CMF                         | 449 (92.6%)                  | 376 (100%)                  |
|                                        | E-CMF                           | 36 (7.4%)                    |                             |
| Adjuvant HT                            | Yes                             | 370 (78.1%)                  | 280 (76.3%)                 |
|                                        | No                              | 104 (21.9%)                  | 87 (23.7%)                  |
| Adjuvant RT                            | Yes                             | 365 (77.7%)                  | 279 (77.1%)                 |
|                                        | No                              | 105 (22.3%)                  | 83 (22.9%)                  |
| ER/PgR status                          | Negative                        | 127 (26.2%)                  | 93 (24.7%)                  |
|                                        | Positive                        | 357 (73.8%)                  | 283 (75.3%)                 |
| HER2                                   | Negative                        | 362 (75.4%)                  | 280 (75.1%)                 |
|                                        | Positive (3+ or ampl)           | 118 (24.6%)                  | 93 (24.9%)                  |
| K67                                    | High (≥14%)                     | 338 (70.1%)                  | 252 (67.4%)                 |
|                                        | Low (<14%)                      | 144 (29.9%)                  | 122 (32.6%)                 |
| TOP2A (FISH)                           | Deleted                         | 26 (5.4%)                    | 18 (4.8%)                   |
|                                        | Non-amplified                   | 399 (82.2%)                  | 311 (82.8%)                 |
|                                        | Amplified                       | 54 (11.2%)                   | 43 (11.4%)                  |
|                                        | Equivocal                       | 6 (1.2%)                     | 4 (1%)                      |
| TOP2A (FISH) binary                    | Amplified                       | 54 (11.2%)                   | 43 (11.4%)                  |
|                                        | Non-amplified                   | 431 (88.8%)                  | 333 (88.6%)                 |
| Subtypes                               | Luminal A                       | 110 (23.0%)                  | 95 (25.5%)                  |
|                                        | Luminal B                       | 184 (38.5%)                  | 139 (37.3%)                 |
|                                        | Luminal-HER2                    | 57 (11.9%)                   | 46 (12.3%)                  |
Table 1. Cont.

| Parameter | Categories | 5 genes* (HE10/00 & HE10/97) | All 14 genes (HE10/00 only) |
|-----------|------------|-----------------------------|-----------------------------|
|           |            | S (HE10/00) | S (HE10/97) | All (HE10/00) | All (HE10/97) |
| HER2-enriched | 60 (12.6%) | 67 (14.0%) | 47 (12.6%) | 46 (12.3%) |
| TNBC       |            |              |              |              |

*according to REMARK (Figure 1);
NOS2, ERBB2, THRA, RARA, TOP2A; MRM = modified radical mastectomy; BCS = breast conserving surgery;
**E = epirubicin; T = paclitaxel (Taxol); C = cyclophosphamide; M = methotrexate; F = fluorouracil.

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Chr17 CN with qPCR

To be able to directly compare classic FISH and qPCR results, DNA was extracted from TMA cores instead of whole tumor sections. DNA was extracted manually with the VERSANT Tissue Preparation Reagents kit (Siemens Healthcare, Erlangen, Germany) [23]. Only cases represented with >50% tumor cells on core sections, as determined by a certified pathologist (M.B.); were included in this study [Figure 1]. With this restriction, of the originally 1027 tumors that had been processed on TMAs from the HE10/00 and HE10/97 series, 508 cases were eligible for DNA extraction, 427 from the HE10/00 and 81 from the HE10/97 trial. CN was assessed for DNA samples from non-cancer patients were included in each run as calibrator samples, along with no-template controls (NTC). Results were obtained automatically with the CopyCaller Software v2.0, as predicted copy numbers in comparison to averaged calibrator values, upon setting the evaluation threshold at CT = 33 for reference RNase P in each reaction. Samples with reference CT>33 were excluded from analysis. Z-scores for all accepted samples and CN range for replicates were <1. No amplification curves were observed for NTCs.

Evaluation of qPCR CN

CopyCaller predicted CN for each gene were initially evaluated separately for the 5’ and 3’ amplicons [Table S2] and analyzed as (a) categorical and (b) continuous variables. For (a), CN were classified into a 5-scale variable as follows: loss: 0.5–1 copies; normal: 1–2 copies; marginal: 3–4 copies; low gains: 5–6 copies; high gains: >6 copies. Because of significant differences in the obtained classification for the 5’ and 3’ ends for some genes, gene status was finally classified according to maximal CN. This compromise was necessary in order to obtain one single status per gene; it was preferred over getting the average of two values for amplicons located distantly to each other in long genes, since structural aberrations cannot be tested with this method. The number of informative cases per gene amplicon and the final number of tumors eligible for single gene analysis is shown in Figure S1. Out of 508 tumors from HE10/00 and HE10/97, results for at least one gene were obtained in 485 cases (95.5%), 411 of which were derived from the HE10/00 series.

For (b), CN values were transformed with the natural logarithm (neper logarithm = 2.78). Log transformation was considered necessary because of the broad range and right skewness of the CN values observed for some genes [Figure S1, Table S1]; the natural log was preferred because it provides values closer to median. The distribution of these values is shown in Figure S2. Again, for log transformation, single gene status as described above, as well as 5’ and 3’ amplicon CN were used. Log transformed CN values were subjected to unsupervised hierarchical clustering with the JMP v8.0 software (SAS Institute Inc., Cary, NC), using the Ward’s minimum variance method. The clusters identified with this approach were used as categorical variables for comparisons with clinicopathological variables and patient out-
Table 2. Concordance between FISH and qPCR CN classification of chromosome 17 gene status.

| Gene     | Non-amplified | Amplified | Kappa Value | Kappa 95% CI | P-value (McNemar)* |
|----------|---------------|-----------|-------------|--------------|-------------------|
| **HER2 FISH status by ratio** |
| TP53     | no gain       | 264 (86.8)| 91 (91.0)   | -0.0500      | (-0.132 to 0.031) | <0.001            |
|          | gain          | 40 (13.2) | 9 (9.0)     |              |                   |                   |
| MAP2K4   | no gain       | 301 (99.7)| 96 (96.0)   | 0.0538       | (-.002 to 0.110)  | <0.001            |
|          | gain          | 1 (0.3)   | 4 (4.0)     |              |                   |                   |
| NOS2     | no gain       | 302 (83.9)| 82 (71.9)   | 0.1289       | (0.032 to 0.226)  | 0.052             |
|          | gain          | 58 (16.1) | 32 (28.1)   |              |                   |                   |
| STARD3   | no gain       | 179 (58.9)| 6 (6.0)     | 0.3779       | (0.305 to 0.450)  | <0.001            |
|          | gain          | 125 (41.1)| 94 (94.0)   |              |                   |                   |
| ERBB2    | no gain       | 322 (89.4)| 22 (19.3)   | 0.6694       | (0.593 to 0.746)  | 0.052             |
|          | gain          | 38 (10.6) | 92 (80.7)   |              |                   |                   |
| PSMD3    | no gain       | 295 (96.7)| 52 (52.0)   | 0.5207       | (0.420 to 0.621)  | <0.001            |
|          | gain          | 10 (3.3)  | 48 (48.0)   |              |                   |                   |
| THRA     | no gain       | 340 (95.0)| 67 (58.3)   | 0.4292       | (0.331 to 0.527)  | <0.001            |
|          | gain          | 18 (5.0)  | 48 (41.7)   |              |                   |                   |
| CDC6     | no gain       | 267 (87.5)| 75 (75.0)   | 0.1432       | (0.039 to 0.247)  | 0.001             |
|          | gain          | 38 (12.5) | 25 (25.0)   |              |                   |                   |
| RARA     | no gain       | 349 (97.2)| 100 (87.7)  | 0.1300       | (0.047 to 0.213)  | <0.001            |
|          | gain          | 10 (2.8)  | 14 (12.3)   |              |                   |                   |
| TOP2A    | no gain       | 343 (96.3)| 106 (93.0)  | 0.0466       | (-.023 to 0.116)  | <0.001            |
|          | gain          | 13 (3.7)  | 8 (7.0)     |              |                   |                   |
| IGFBP4   | no gain       | 270 (89.1)| 85 (85.0)   | 0.0498       | (0.039 to 0.144)  | <0.001            |
|          | gain          | 33 (10.9) | 15 (15.0)   |              |                   |                   |
| SMARCE1  | no gain       | 270 (88.2)| 83 (83.8)   | 0.0524       | (0.044 to 0.149)  | <0.001            |
|          | gain          | 36 (11.8) | 16 (16.2)   |              |                   |                   |
| KRT20    | no gain       | 279 (91.5)| 90 (91.8)   | -0.005       | (-.085 to 0.076)  | <0.001            |
|          | gain          | 26 (8.5)  | 8 (8.2)     |              |                   |                   |
| STAT3    | no gain       | 268 (88.2)| 97 (98.0)   | -0.124       | (-.178 to -.070)  | <0.001            |
|          | gain          | 36 (11.8) | 2 (2.0)     |              |                   |                   |
| **TOP2A FISH status by ratio** |
| TP53     | no gain       | 295 (88.1)| 60 (87.0)   | 0.0125       | (-.086 to 0.110)  | 0.057             |
|          | gain          | 40 (11.9) | 9 (13.0)    |              |                   |                   |
| MAP2K4   | no gain       | 330 (99.1)| 67 (97.1)   | 0.0316       | (-.033 to 0.096)  | <0.001            |
|          | gain          | 3 (0.9)   | 2 (2.9)     |              |                   |                   |
| NOS2     | no gain       | 329 (83.5)| 55 (68.8)   | 0.1405       | (0.038 to 0.243)  | 0.411             |
|          | gain          | 65 (16.5) | 25 (31.3)   |              |                   |                   |
| STARD3   | no gain       | 172 (51.3)| 13 (18.8)   | 0.1745       | (0.108 to 0.241)  | <0.001            |
|          | gain          | 163 (48.7)| 56 (81.2)   |              |                   |                   |
| ERBB2    | no gain       | 318 (80.7)| 26 (32.5)   | 0.3860       | (0.292 to 0.480)  | <0.001            |
|          | gain          | 76 (19.3) | 54 (67.5)   |              |                   |                   |
| PSMD3    | no gain       | 304 (90.5)| 43 (62.3)   | 0.3006       | (0.180 to 0.421)  | 0.248             |
|          | gain          | 32 (9.5)  | 26 (37.7)   |              |                   |                   |
| THRA     | no gain       | 368 (93.6)| 39 (48.8)   | 0.4825       | (0.374 to 0.591)  | 0.103             |
|          | gain          | 25 (6.4)  | 41 (51.3)   |              |                   |                   |
| CDC6     | no gain       | 304 (90.5)| 38 (55.1)   | 0.3867       | (0.248 to 0.486)  | 0.550             |
|          | gain          | 32 (9.5)  | 31 (44.9)   |              |                   |                   |
| RARA     | no gain       | 386 (98.2)| 63 (78.8)   | 0.2699       | (0.156 to 0.384)  | <0.001            |
|          | gain          | 7 (1.8)   | 17 (21.3)   |              |                   |                   |
| TOP2A    | no gain       | 378 (96.9)| 71 (88.8)   | 0.1156       | (0.018 to 0.214)  | <0.001            |
come. Clustering with the 14-gene set was informative in 376 of 416 HE10/00 samples (90%).

The demographic and clinicopathological characteristics for patients with informative tumors in (a) and (b) are shown in Table 1.

Statistics

Categorical data are presented as numbers and corresponding percentages, continuous data as median and range values. Fisher’s exact or Pearson’s chi-square tests were used for group comparisons of categorical data; non-parametric Spearman’s correlation was used for the evaluation of continuous CN in paired gene comparisons, whereby rho (correlation coefficient) values > 0.5 were considered to be significant. With respect to continuous Ki67 IHC data, ROC-curve analysis with DFS and OS at 5-years, as clinical endpoints, did not prove helpful for setting a specific cut-off in HER2-positive and triple-negative tumors; hence, the 14% Ki67 cut-off was used for all tumors.

Concordance between HER2 and TOP2A FISH and CN status was evaluated with (a) the Kappa coefficient, whereby Kappa of 1 corresponds to complete agreement, between 0.75 and 1 to strong, between 0.4 and 0.75 to fair, and, <0.4 to poor agreement; (b) the McNemar test for comparing agreement between binary FISH (negative vs. positive) and CN values (no gains vs. gains); and (c) multiple correspondence analysis (MCA) for drawing conclusions about the underlying relationship between FISH data and gene CN.

Disease-free survival (DFS) was measured from the date of diagnosis until verified disease progression, death or last contact, whichever occurred first, and overall survival (OS) from diagnosis until death from any cause or date of last contact. Time-to-event distributions were estimated using the product limit method. Kaplan-Meier curves and log-rank tests were used for comparing time to event distributions and evaluating DFS and OS differences, while univariate Cox regression analysis was used for reporting hazard ratios. Cox regression was also applied for testing

Table 2. Cont.

| Gene | Non-amplified | Amplified | Kappa Value | Kappa 95% CI | P-value (McNemar)* |
|------|---------------|-----------|-------------|--------------|-------------------|
| HER2 FISH status by ratio | | | | | |
| gain | 12 (3.1) | 9 (11.3) | 0.2343 | (0.113 to 0.355) | 0.022 |
| IGFBP4 | no gain | 306 (91.6) | 49 (71.0) | 0.2157 | (0.096 to 0.335) | 0.075 |
| gain | 28 (8.4) | 20 (29.0) | | | |
| SMARCE1 | no gain | 304 (90.5) | 49 (71.0) | 0.2157 | (0.096 to 0.335) | 0.075 |
| gain | 32 (9.5) | 20 (29.0) | | | |
| KRT20 | no gain | 309 (92.5) | 60 (87.0) | 0.0696 | (<0.05 to 0.174) | <0.001 |
| gain | 25 (7.5) | 9 (13.0) | | | |
| STAT3 | no gain | 305 (91.3) | 60 (87.0) | 0.0531 | (<0.05 to 0.156) | 0.001 |
| gain | 29 (8.7) | 9 (13.0) | | | |

Non-amplified: HER2/CEP17 ratio < 2.2, TOP2A/CEP17 ratio < 2;

*McNemar higher P-values indicate higher agreement.
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![Figure 3. Multiple Correspondence Analyses graphs for comparing gene copies as assessed with qPCR and FISH. A: Correspondence of HER2 FISH (copies) to CN variables (n = 398). B: Correspondence of TOP2A FISH (copies) with CN variables co-expression (n = 393). Green: normal or loss; red: gain or amplification.
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interactions between the identified clusters and standard clinicopathological parameters, i.e., effects produced on outcome by one variable examined according to different levels of the other. In multivariate Cox regression analysis, cluster interactions were adjusted for significance against clinicopathological variables among the following (categories as in Table 1): age, treatment group, menopausal status, histological grade, tumor size, number of positive axillary nodes, Er/PgR and HER2 status, Ki67 IHC, adjuvant hormonotherapy and type of operation. The examined interactions were included in the final model, in order to investigate whether they added independent prognostic information, as compared to the significant clinicopathological parameters. In multivariate analysis, significance was determined at the level of 15% and in univariate at 3%. All tests were two-sided.

The SAS software was used for statistical analysis (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC), while no adjustments for multiple comparisons were reported. Statistical analysis complied with the reporting recommendations for tumor marker prognostic studies [24].

Raw data (gene target per run) including the overview of runs can now be found at http://hecog-images.gr/17CN/.

Results

Chr17 gene CN Strongly correlate with each other within the core HER2 amplicon

The associations between single gene max CN (5-scale, categorical) and standard clinicopathological parameters are shown in Table S3. High CN (gains and amplification) were observed significantly more often in tumors from patients >50 years old for ERBB2 (p = 0.014) and SMARCE1 (p = 0.012); in post-menopausal patients for NOS2 (p = 0.038), ERBB2 (p = 0.015), PSMD3 (p = 0.008), IGFBP4 (p = 0.030) and STAT3 (p = 0.011); in high-grade tumors for STARD3 (p = 0.009), ERBB2 (p = 0.004) and CDC6 (p = 0.022); and, in ER/PgR-negative tumors for STARD3 and ERBB2 (both p < 0.001). High CN for ERBB2 and four additional genes in its proximity (STARD3 centromerically; PSMD3, THRA and CDC6 telomerically) strongly coincided with positive classic HER2 status, as expected (all p’s <0.001). High CN for the same genes coincided with amplified TOP2A by FISH, but unexpectedly, RARA, TOP2A and IGFBP4, i.e., the genes covered by the TOP2A FISH probe, did not.

CN for ERBB2 and its neighbor genes (STARD3, PSMD3, THRA) strongly correlated with each other with all analytical approaches, i.e., as 5’ and 3’ end results in the 5-scale classification (Figure S3) or as continuous log-transformed values (Figure 2). CN for NOS2, located centromerically to ERBB2, as well as for RARA and IGFBP4 located telomerically to THRA, also strongly correlated to the four HER2-amplicon-related genes and to each other. By contrast, CN for the TOP2A gene, which is located between RARA and IGFBP4, vaguely or minimally correlated with these two and with HER2-related genes. Similarly, the CN status for CDC6, which is located between THRA and RARA, was closer related to TOP2A than to any other gene tested.

Lack of concordance between TOP2A FISH status and qPCR CN

The lack of TOP2A status correlation with its neighboring genes was unexpected. For the evaluation of qPCR CN against HER2 and TOP2A FISH status, individual gene CN status was evaluated in a binary mode as “gain” for >4 maximal copies per gene, which was expected to correspond to gene amplification by FISH assessed as gene/CEN17 ratio or >6 copies. With ratios, FISH HER2 status was concordant with ERBB2, PSMD3 and THRA CN, based on Kappa values; based on both Kappa and McNemar results, concordance was strongest between HER2 FISH and ERBB2 (Table 2). TOP2A CN was not concordant with TOP2A FISH, the latter mainly corresponding to THRA CN status. Similar results were obtained when assessing qPCR CN status against HER2 and TOP2A FISH copies. With multiple correspondence analysis, HER2 amplified cases with FISH coincided with those exhibiting >4 CN with qPCR for ERBB2, STARD3, PSMD3 and THRA; in comparison, cases called as TOP2A amplified with FISH were not identified as harboring TOP2A CN gains with qPCR (Figure 3). TOP2A FISH amplified cases had high CN for ERBB2, PSMD3, THRA, CDC6 and IGFBP4.

Patterns of chr17 gene CN

Since chr17 gene CN aberrations did not occur singly but were related to each other, we next examined CN patterns. Hierarchical clustering of log transformed CN for the 14 genes (Figure 4A) revealed four main clusters based on the distance dendrogram. These clusters distinguished tumors with HER2-related gains, non-HER2-related gains, low CN (normal or losses), and complex changes. The cluster with HER2-related gains almost exclusively included HER2-positive tumors (luminal-HER2 and HER2-enriched) prompting for separate analysis of HER2-related and non-HER2 CN patterns in order to avoid HER2 bias. HER2-related gene CN clustering revealed STARD3, ERBB2, PSMD3 and THRA CN gains, complex changes and normal status (including possible losses) in three distinct groups (Figure 4B). Similarly, clustering of the remaining 10 non-HER2-related gene CN, again yielded three main patterns with gains, complex changes, and normal/losses (Figure 4C).

HER2-related gene CN clusters showed the expected strong associations with menopausal and tumor hormonal status, histological grade, tumor HER2 and TOP2A FISH status, and, with HER2-positive subtypes (Table 3). Non-HER2-related complex CN changes were significantly more often in larger (39/109, 52.3%) as compared to smaller tumors (138/264, 35.8%); while gains were less often in hormone receptor positive (52/284, 18.3%) as compared to negative tumors (34/89, 38.2%). Non-HER2-related clusters showed a similar distribution in luminal tumors, which differed from that observed in HER2-enriched and triple-negative breast cancer (TNBC).

Figure 4. Hierarchical clustering of log transformed qPCR copy number (CN) values for chr17 genes in association with relevant breast cancer subtypes. A: clustering of all 14 genes was informative in 376 cases and revealed 4 main CN patterns, out of which high CN for the HER2-related genes form the HER2 cluster and are predominantly found in HER2-positive (Luminal-HER2 and HER2-enriched) tumors; gains for the HER2 gene itself (ERBB2) mostly occur in parallel with STARD3. Luminal A and B, as well as TNBC are evenly represented in the non-HER2 clusters. B: if analyzing CN for the 4 HER2-related genes only, 36 of 93 tumors (38.7%) have non-continuous gains for these genes (complex pattern, red cluster) while the rest exhibit continuous gains in at least two genes, predominantly STARD3 and ERBB2 (gains, green cluster). C: Clustering of the non-HER2-related gene CN, i.e., 10 of 14 genes excluding STARD3, ERBB2, PSMD3 and THRA, yields 3 main clusters with equal representation of all subtypes (distribution of HER2-positive tumors and TNBC is shown). Cluster legend: natural log 0.69 corresponds to 2 copies; log 1.38 to 4 copies. doi:10.1371/journal.pone.0103707.g004
Table 3. HER2- and non-HER2-related gene CN clusters comparisons with standard clinicopathological parameters.

|                       | Non-HER2 gene CN clusters | HER2-related gene CN clusters |
|-----------------------|---------------------------|------------------------------|
|                       | Gains | Complex | Normal/Losses | P-value | HER2amp | Complex | Normal/Losses | P-value |
| Total cluster N = 376 | 92 (24.47) | 178 (47.34) | 106 (28.19) | 55 (14.63) | 166 (44.15) | 153 (40.69) |
| **Age**               |       |         |               |         |         |         |               |         |
| 34–50                 | 26 (30.2) | 63 (35.6) | 34 (30.9) | 0.592 | 16 (32.0) | 54 (30.7) | 66 (38.4) | 0.106 |
| <34                   | 2 (2.3) | 5 (2.8) | 6 (5.5) |         | 4 (2.3) | 9 (5.2) |         |         |
| >50                   | 58 (67.4) | 109 (61.6) | 70 (63.6) |         | 34 (68.0) | 118 (67.0) | 97 (56.4) |         |
| **Menopausal Status** |       |         |               |         |         |         |               |         |
| Post                  | 60 (69.8) | 103 (58.2) | 64 (58.2) | 0.155 | 33 (66.0) | 117 (66.5) | 88 (51.2) | 0.009 |
| Pre                   | 26 (30.2) | 74 (41.8) | 46 (41.8) |         | 17 (34.0) | 59 (33.5) | 84 (48.8) |         |
| **Type of surgery**   |       |         |               |         |         |         |               |         |
| BCS (conserving)      | 34 (39.5) | 60 (33.9) | 42 (38.2) | 0.609 | 16 (32.0) | 63 (35.8) | 68 (39.5) | 0.571 |
| MRM (modified radical) | 52 (60.5) | 117 (66.1) | 68 (61.8) |         | 34 (68.0) | 113 (64.2) | 104 (60.5) |         |
| **Tumor size**        |       |         |               |         |         |         |               |         |
| ≤2 cm                 | 29 (33.7) | 39 (22.0) | 41 (37.3) | 0.013 | 13 (26.0) | 49 (27.8) | 54 (31.4) | 0.668 |
| >2 cm                 | 57 (66.3) | 138 (78.0) | 69 (62.7) |         | 37 (74.0) | 127 (72.2) | 118 (68.6) |         |
| **Number of positive nodes** |       |         |               |         |         |         |               |         |
| 1–3                   | 43 (50.0) | 80 (45.2) | 47 (42.7) | 0.592 | 23 (46.0) | 85 (48.3) | 76 (44.2) | 0.744 |
| >3                    | 43 (50.0) | 97 (54.8) | 63 (57.3) |         | 27 (54.0) | 91 (51.7) | 96 (55.8) |         |
| **Histological grade**|       |         |               |         |         |         |               |         |
| I–II                  | 39 (45.3) | 75 (42.4) | 55 (50.0) | 0.451 | 14 (28.0) | 74 (42.0) | 93 (54.1) | 0.002 |
| III-Undifferentiated   | 47 (54.7) | 102 (57.6) | 55 (50.0) |         | 36 (72.0) | 102 (58.0) | 79 (45.9) |         |
| **Histological type** |       |         |               |         |         |         |               |         |
| Comedo                | 1 (1.2) | 7 (4.0) | 2 (1.8) | 0.473 | 2 (4.0) | 2 (1.1) | 7 (4.1) | 0.180 |
| Inflammatory          | 1 (0.6) | 1 (0.6) | 2 (1.8) |         | 2 (1.1) | 1 (0.6) |         |         |
| Invasive ductal       | 73 (84.9) | 143 (80.8) | 85 (77.3) |         | 46 (92.0) | 147 (83.5) | 127 (73.8) |         |
| Invasive lobular      | 7 (8.1) | 16 (9.0) | 9 (8.2) |         | 2 (4.0) | 14 (8.0) | 18 (10.5) |         |
| Medullary             | 2 (2.3) | 1 (0.6) | 4 (3.6) |         | 2 (1.1) | 6 (3.5) |         |         |
| Mixed                 | 3 (3.5) | 9 (5.1) | 8 (7.3) |         | 9 (5.1) | 12 (7.0) |         |         |
| Papillary             |   1 (0.6) |         |         |         |         |         |         |         |
| **ER/PgR status**     |       |         |               |         |         |         |               |         |
| Negative              | 34 (39.5) | 29 (16.4) | 26 (23.6) | <0.001 | 32 (64.0) | 44 (25.0) | 20 (11.6) | <0.001 |
| Positive              | 52 (60.5) | 148 (83.6) | 84 (76.4) |         | 18 (36.0) | 132 (75.0) | 152 (88.4) |         |
| **HER2 status**       |       |         |               |         |         |         |               |         |
| Negative              | 62 (72.1) | 135 (77.1) | 79 (72.3) | 0.565 | 5 (10.0) | 128 (73.6) | 162 (94.7) | <0.001 |
| Positive (3+ or amplified) | 24 (27.9) | 40 (22.9) | 30 (27.5) |         | 45 (90.0) | 46 (26.4) | 9 (5.3) |         |
| Table 3. Cont. | Non-HER2 gene CN clusters | HER2-related gene CN clusters |
|---------------|---------------------------|-------------------------------|
|               | Gains | Complex | Normal/Losses | P-value | HER2amp | Complex | Normal/Losses | P-value |
| **Ki67**      |       |         |               |         |         |         |               |         |
| High (≥14%)   | 52 (61.2) | 121 (68.4) | 79 (72.5) | 0.243 | 35 (71.4) | 127 (72.2) | 105 (61.4) | 0.083 |
| Low (<14%)    | 33 (38.8) | 56 (31.6)  | 30 (27.5)  |         | 14 (28.6) | 49 (27.8)  | 66 (38.6)  |         |
| **TOP2A (FISH)** |     |         |               |         |         |         |               |         |
| Amplified     | 16 (18.6) | 17 (9.6)  | 10 (9.1)    | 0.128 | 24 (48.0) | 17 (9.7)   | 5 (2.9)    | 0.000 |
| Deleted       | 5 (5.8)  | 6 (3.4)   | 8 (7.3)     |         | 7 (14.0) | 5 (2.8)    | 8 (4.7)    |         |
| Equivocal     | 2 (2.3)  | 1 (0.6)   | 1 (0.9)     |         | 3 (1.7)  | 1 (0.6)    |           |         |
| Non-amplified | 63 (73.3) | 153 (86.4) | 91 (82.7)  |         | 19 (38.0) | 151 (85.8) | 158 (91.9) |         |
| **TOP2A (FISH) binary** |       |         |               |         |         |         |               |         |
| Amplified     | 16 (18.6) | 17 (9.6)  | 10 (9.1)    | 0.064 | 24 (48.0) | 17 (9.7)   | 5 (2.9)    | <0.001 |
| Non-amplified | 70 (81.4) | 160 (90.4) | 100 (90.9) |         | 26 (52.0) | 159 (90.3) | 167 (97.1) |         |
| **Subtypes (IHC4)** |     |         |               |         |         |         |               |         |
| Luminal A     | 24 (27.9) | 46 (26.3) | 24 (22.0)   | 0.006 | 1 (2.0)  | 40 (23.0)  | 61 (35.7)  | <0.001 |
| Luminal B     | 21 (24.4) | 76 (43.4) | 42 (38.5)   |         | 1 (2.0)  | 64 (36.8)  | 81 (47.3)  |         |
| Luminal HER2   | 7 (8.1)  | 24 (13.7) | 17 (15.6)   |         | 16 (32.0) | 26 (14.9)  | 9 (5.3)    |         |
| HER2-enriched | 17 (19.8) | 16 (9.1)  | 13 (11.9)   |         | 29 (58.0) | 20 (11.5)  |           |         |
| TNBC          | 17 (19.8) | 13 (7.4)  | 13 (11.9)   |         | 3 (6.0)  | 24 (13.8)  | 20 (11.7)  |         |
| **Adjuvant RT** |     |         |               |         |         |         |               |         |
| No            | 23 (27.1) | 37 (22.3) | 24 (22.2)   | 0.659 | 11 (23.4) | 42 (24.4)  | 37 (22.4)  | 0.911 |
| Yes           | 62 (72.9) | 129 (77.7)| 84 (77.8)   |         | 36 (76.6)| 130 (75.6)| 128 (77.6) |         |
| **Adjuvant HT** |     |         |               |         |         |         |               |         |
| No            | 22 (25.6) | 31 (18.2) | 27 (25.2)   | 0.260 | 20 (40.0) | 43 (24.9)  | 27 (16.4)  | 0.002 |
| Yes           | 64 (74.4) | 139 (81.8)| 80 (74.8)   |         | 30 (60.0)| 130 (75.1)| 138 (83.6) |         |
Figure 5. Significant interactions between non-HER2 gene CN clusters and clinicopathologic parameters affecting patient survival.

In A, among patients with non-TNBC tumors those with non-HER2 gene CN gains fare best; among patients with TNBC, those with non-HER2 gains fare worse. In B, patients with favorable nodal status (1–3 metastatic lymph nodes) and tumors with non-HER2-gene CN losses have similarly bad outcome as those with unfavorable nodal status. In C, non-HER2-gene CN gains strikingly confer the opposite outcome to patients who have undergone modified radical mastectomy (MRM) as compared to breast-conserving surgery (BCS).

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Figure 6. Multivariate analysis models involving the main significant non-HER2-gene CN genotype effects on patient DFS. Red circles: novel findings with potentially significant clinical implications, worthy pursuing for further validation. CN gains in non-HER2 genes on chr17 confer inverse risk for relapse in patients with TNBC and non-TNBC tumors, and in those who have undergone BCS in comparison to MRM. Similarly, the favorable 1–3 nodal status confers the same risk for relapse as the unfavorable disease with >3 positive nodes, if tumors have non-HER2-gene CN losses.
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Figure 7. Multivariate analysis models involving the main significant non-HER2-gene CN pattern effects on patient OS. Findings as for DFS in Figure 6.
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Diverse effects of the same gene CN patterns on patient outcome according to tumor subtype, nodal status and the applied type of surgery

DFS and OS did not differ significantly between treatment groups. At a median follow-up of 101.7 months (range: 0.1–161.4), the 5-year DFS rates were 77.1% and 69.3%, while the OS rates were 88.9% and 83.2%, for the E-T-CMF and E-CMF arms, respectively. Clinicopathological parameters significantly implicated in patient outcome, as revealed by log-rank testing for OS, were type of surgery (favorable for BCS, p = 0.0498), nodal status (favorable for 1–3 positive nodes, p = 0.0003), and IHC4 subtypes (worst for TNBC, p = 0.0098); type of surgery (p = 0.0389) and nodal status (p < 0.001) for DFS. Single gene CN status (3 scale) was not associated with patient outcome, neither were CN clusters with all 14 genes or with the 10 non-HER2-related genes. Tumors associated with favorable outcome as compared to MRM only in 95% CI 0.3–1.0; OS HR 0.4, 95% CI 0.2–0.9). BCS was a favorable outcome to those who underwent MRM (DFS HR 2.1, 95% CI 1.1–4.3; OS HR 2.6, 95% CI 1.0–6.6) but unfavorable outcome among patients with 1–3 metastatic nodes, p = 0.0082 for DFS; p = 0.0092 for OS) (Figure 5A). Among patients in the favorable prognosis group with 1–3 positive nodes, the outcome of those with non-HER2-related CN losses was in fact unfavorable (DFS HR 2.9, 95% CI 1.5–5.6; OS, HR 5.4, 95% CI 2.1–13.8), similar to the outcome of patients with >3 positive nodes and tumors with any other non-HER2-related CN pattern (interaction p = 0.0172 for DFS; p = 0.0010 for OS) (Figure 5B).

The described non-HER2-related CN cluster interactions remained significant upon multivariate analysis for both DFS and OS (Figure 6). As shown, non-TNBC and TNBC lacking CN gains within this group of 10 genes shared similar outcomes, while the unfavorable behavior of TNBC was independently predicted by the presence of CN gains of the same genes. Low non-HER2-gene CN independently predicted for an unfavorable outcome among patients with 1–3 metastatic nodes, which did not differ from that of patients with >3 metastatic nodes. And, the presence of non-HER2-related CN gains with respect to BCS and MRM independently predicted for an unfavorable and favorable outcome, respectively.

Discussion

In accordance to previous reports involving assessment of multiple chr17 genes or regions, this study once more portrays the complexity of chr17 alterations in breast cancer. There was no single tumor with CN gains for all 14 genes, further supporting the rarity of chr17 polysomy, i.e., increased copies of the entire chromosome, as also shown with array-based comparative genomic hybridization (aCGH) [4,6,7], alternative FISH approaches [11] and MLPA [25]. In order to compare the present data on gene dosage with relevant literature findings, however, we need to consider technical characteristics of the methods applied for this purpose. qPCR targets very short amplicons (up to 200 bp) within individual genes. In comparison, the BAC probes used for FISH and aCGH span hundreds of kilobases. Therefore, results by FISH and aCGH for chr17 may be highly concordant [26], but qPCR CN merely delineate those by aCGH [27]. Further, differences in the number, selection and application of reference genes for predicting CN, type of probes used, as well as differences in the selection of the amplicons targeted with qPCR may also yield different results with various qPCR approaches. The above explain, for example, why HER2 FISH and qPCR ERBB2 CN status were only fairly concordant in the present study, as compared to the strong concordance reported with a different qPCR approach [28].

In line with the only study so far, using qPCR for assessing the status of multiple genes on 17q [8], CN of the genes surrounding ERBB2, i.e., STARD3, PSMD3 and THRA mostly increased in parallel. STARD3 and ERBB2 exhibited the highest CN values observed in this study, mostly but not always coinciding. Thus, STARD3 may rather be at the edge of the smallest region of amplification described for the HER2 amplicon [29] and not within this region as stated by others [8,30–32]. STARD3, a gene involved in steroidogenesis [33], is overexpressed in HER2-positive cancer cells [34] and it seems worthy investigating whether it contributes to the lipid-rich phenotype described for HER2-positive tumors [35]. THRA, another metabolic gene experimentally shown to interfere with insulin resistance [36], was also frequently co-amplified along with ERBB2 and STARD3. Hence, STARD3 and THRA may represent additional targets for therapeutic interventions in HER2-positive cancers.

Unexpectedly, qPCR TOP2A CN were not associated with TOP2A FISH, HER2 FISH and ERBB2 CN status, which may partially explain the discrepancy between FISH TOP2A amplification and respective mRNA and protein expression described partially for this series [37] and elsewhere [38]. Instead, TOP2A CN did associate with THRA and CDC6 CN. Concordance of TOP2A FISH has been described for qPCR with hybridization probes [39] but respective reports with hydrolysis (Taqman) probes are particularly missing. With classic FISH, TOP2A amplification is rare in HER2 non-amplified tumors, which was noticed for the present series, as well. However, TOP2A gene gains with qPCR have been independently reported as unrelated to HER2 FISH status [40,41], in line with the present findings. This discrepancy may be explained by (a) the presence of breakpoints and possible rearrangements within the 17q21 region covered by the FISH probe targeting RARA and IGFBP4 in addition to TOP2A; (b) the break-fusion-bridge pattern, proposed for the development of TOP2A amplification, has been suggested by using probes covering the THRA region, as well [42]; (c) the use of ratios instead of copies with FISH; and, (d) the different specificity/sensitivity of the TOP2A FISH assays [43]. Overall, due to the low number of TOP2A copies observed with both FISH [44] and qPCR, the use of gene ratios vs. the centromeric probe should probably be replaced by gene copies when evaluating amplification, as was recently also suggested for the evaluation of the HER2 gene with FISH [45].

Considering that the CN clusters reflect different levels and types of chr17 instability, this study supports the notion that...
HER2-positive tumors exhibit different patterns of CIN [46]. Herein we found that compact HER2 amplicon gains are more common in HER2-enriched, while complex gains are the main pattern in luminal-HER2 tumors. Interestingly, HER2-positive patients with compact HER2 amplicon gains had similar time-to-relapse, but significantly prolonged survival as compared to patients with normal or complex HER2 amplicon gene CN. Since all HER2-positive patients had received trastuzumab upon relapse, the observed favorable survival for those with the compact gains pattern might be attributed to this drug, as seen in patients with high level HER2 gene amplification [47] and HER2-enriched tumors [48].

The different CN patterns of the 10 genes outside the core HER2 amplicon may reflect the presence of various degrees and patterns of CIN among breast cancer subtypes, as observed with different methods [6,7,49–51]. The distribution of these patterns differed in luminal and non-luminal subtypes, indicating an estrogen-related background playing a role in the development of these complex patterns, and supporting previous evidence that the luminal B subtype is the most unstable one among ER-positive tumors [49]. Importantly, the obtained chr17 CN patterns predicted for good prognosis in patients with standard unfavorable characteristics and vice versa. Thus, the favorable prognosis in patients with 1 to 3 metastatic lymph nodes was observed only in those with gains and complex CN patterns of non-HER2 genes. By contrast, prognosis for patients with the same favorable nodal status, but with tumors exhibiting predominantly losses of non-HER2 genes, was similar to patients with unfavorable nodal status. This seems to contradict a previous report on unstable tumors with negative nodal status faring worse [51]. However, the present chr17 patterns are not comparable to the genomic index in the latter study, while in the present series, there were no patients with negative nodal status. Hence, the two studies actually show that different patterns of instability have diverse effects on the outcome of patients with the same nodal status.

The impact of the same chromosomal stability status, as determined by different methods [49,50,52], was inversely related to the outcome of patients with ER-positive as compared to ER-negative tumors, the behavior of which is driven by different pathways [53]. In a similar manner, among the 5 subtypes considered in the present study, TNBC patients had overall the worst outcome, which concerned tumors with the chr17 non-HER2 CN gain pattern only. In fact, TNBC patients with losses or complex non-HER2 CN patterns had a relatively more favorable outcome, similar to non-TNBC, mostly ER-positive patients with chr17 non-HER2 gains in their tumors. To our knowledge this is a novel finding that would be worth validating in larger patient series; distinguishing between favorable and unfavorable TNBC, based on chr17 or genome wide CIN patterns, might aid in modifying treatment selection accordingly.

In addition, with respect to the applied surgery type, BCS was overall associated with better survival, which was recently presented as an effect of nodal status on long term follow-up [54]. Here we show that the outcome of patients who underwent BCS or MRM may independently be predicted by the chr17 CN pattern in the tumor. Patients with tumors exhibiting chr17 non-HER2 losses or complex patterns and patients with chr17 gains shared the same favorable outcome on BCS and MRM. By contrast, the same unfavorable outcome was observed for patients who received BCS for tumors with chr17 gains and MRM for tumors with chr17 losses or complex patterns. This is also a novel finding with potential clinical relevance, which, if validated, would prompt for core needle biopsy before surgery. Relevant data exist for biomarker testing in the neoadjuvant setting [55]. It remains questionable though, whether this type of biopsy would be appropriate for small sized tumors, as was the case for about 1/5 of the patients in the present series.

Overall, the herein presented chr17 CN patterns may reflect the presence of different CIN types that probably evolve on the ground of different DNA repair defects. Thus, next to the extent of CIN [1], the type of these changes also seems to affect disease course. Whether chr17 instability is a surrogate for the condition in the whole genome, as previously stated [10], and whether it interferes with anthracyclines or taxanes for patient outcome [1,56] cannot be answered by the present study. It is important to note though, that chr17 CN alterations outside the core HER2 amplicon occur in HER2-negative tumors and interfere with the outcome of breast cancer patients with established prognostic disease characteristics. Re-interpretation of traditionally obtained chr17 gene results with FISH, classification and typing of CIN with appropriately standardized methods and understanding its diverse effects on disease behavior seem mandatory for efficiently assessing patients with high-risk breast cancer.

Supporting Information

Figure S1 qPCR CN 5-scale classification. The number of informative samples and copy number (CN) distribution per category is shown. Genes are in lanes, classification for 5’ and 3’ ampiclons, and for maximal CN is in rows. Red circles: significant discrepant classification between 5’ and 3’ ends.

Figure S2 CN value distribution upon logarithmic transformation (natural logarithm). a, 5’ end; b, 3’ end; c, highest value of the two. CN ranged between 2–4 in the majority of tumors, indicating low gains and/or DNA replication in the majority of cells.

Figure S3 Clustered correlations for 5’ and 3’ end copy number (CN) values and for maximal CN. A, log transformed; B, 5-scale classification. C, maximal CN (5-scale) Similar to log transformed CN in Figure 1, core HER2 amplicon genes (STARD3, ERBB2, PSMD3, THR) cluster together for both ampiclons per gene and for maximal CN. CN for NOS2, RARA and IGFBP4 also correlate to each other, while TOP2A status is only vaguely related to these genes.

Table S1 qPCR assays for the assessment of chromosome 17 gene CN.

Table S2 Description of predicted CN for each assay per gene and of the finally used values (Maximal CN [Max CN]).

Table S3 Max CN associations with clinicopathological parameters.

Table S4 Impact on patient disease-free survival (DFS) of interactions between chromosome 17 gene CN clusters and clinicopathological parameters (Univariate Cox).

Table S5 Impact on patient overall survival (OS) of interactions between chromosome 17 gene CN clusters and clinicopathological parameters (Univariate Cox).
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