Quantification and clinical relevance of gene amplification at chromosome 17q12-q21 in human epidermal growth factor receptor 2-amplified breast cancers

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Abstract

Introduction: Human epidermal growth factor receptor 2 (HER2)-amplified breast cancers represent a tumor subtype with chromosome 17q rearrangements that lead to frequent gene amplifications. The aim of this study was to quantify the amplification of genes located on chromosome 17q and to analyze the relations between the pattern of gene amplifications and the patients’ characteristics and survival.

Methods: Patients with HER2-positive breast tumors (HER2 score of 3+ by immunohistochemistry or positive for HER2 amplification by fluorescence in situ hybridization (FISH)) (n = 86) and with HER2-negative breast tumors (n = 40) (negative controls) were included in this study. Using a quantitative polymerase chain reaction method and DNA extracted from frozen tumor specimens, 11 genes (MED1, STARD3, HER2, GRB7, THRA, RARA, TOP2A, IGFBP4, CCR7, KRT20, KRT19 and GAS), which are localized within Chr17q12-q21 and have a putative role in breast cancer development, were quantified. Relapse-free and overall survival rates were estimated from the date of surgery to the date of the event of interest (recurrence or death) using the Kaplan-Meier method.

Results: Gene amplification was observed only in HER2-positive tumors, and the frequency of amplification decreased with the distance of the gene from HER2. HER2 presented the highest level of amplification. TOP2A was not included in the smallest region of amplification involving HER2. Amplification of RARA, KRT20 and KRT19 was significantly associated with node-positive breast cancer (P = 0.030, P = 0.002 and P = 0.033, respectively). During a median follow-up period of 55 months (range, 6 to 81 months), the subgroup of patients with hormone receptor-negative cancer and without TOP2A amplification showed the worst survival (relapse-free survival: hazard ratio (HR) = 0.29, 95% confidence interval (95% CI), 0.13 to 0.65, P = 0.001; and overall survival: HR = 0.28, 95% CI, 0.10 to 0.76, P = 0.008).

Conclusions: HER2 amplification seems to drive genomic instability along chromosome 17q, leading to different patterns of gene amplification. This study confirms the clinical importance of identifying, among patients with HER2-positive breast tumors, the subgroup of patients with hormone receptor-negative and nonamplified TOP2A cancers as they have the worst prognosis.
Introduction

Gene amplification at chromosome 17q (Chr17q) involves the human epidermal growth factor receptor 2 (HER2) gene and is observed in about 15% to 20% of breast cancers. HER2 amplification is associated with aggressive tumor behavior (increased metastatic potential and reduced survival), and it also predicts a patient’s response to trastuzumab therapy [1,2]. Although HER2-positive breast cancers are classified as a tumor subgroup, they are quite heterogeneous. Indeed, they could be divided more or less equally into hormone receptor-negative (estrogen receptor/progesterone receptor-negative, or ER/PR-negative) and hormone receptor-positive (ER/PR-positive) tumors. In the ER/PR-positive subgroup, development of resistance to antihormonal therapy could be explained by the cross-talk between the HER2 and ER pathways [3]. Furthermore, first-line treatment of HER2-positive breast cancers with trastuzumab presents response rates that vary between 25% and 35%, and the benefit of trastuzumab therapy has been shown only in patients with high levels of HER2 amplification [4]. Comparative genomic hybridization arrays and fluorescence in situ hybridization (FISH) studies have shown that HER2-amplified breast cancers present a variety of alterations on Chr17 and gene amplifications at Chr17q12-q21 are particularly frequent and complex [5]. Among the genes that are located in this region and have been reported to be amplified in breast cancers, topoisomerase II-a (TOP2A), a gene coding for the enzyme targeted by anthracyclines, is located 2 Mb from HER2. TOP2A amplification might be linked to the sensitivity to anthracyclines observed in HER2-positive breast tumors [6-18]. Most of the published data indicate that TOP2A amplification occurs only in HER2-amplified tumors. However, some authors have reported cases in which TOP2A was amplified also in the absence of HER2 amplification [19,20]. Interestingly, the same studies do not support the role of TOP2A amplification as a predictive marker for response to anthracyclines. These works also report a different frequency of TOP2A amplifications and deletions. The presence of TOP2A deletions, often analyzed together with TOP2A amplification, has complicated the question of the relations among TOP2A alteration, prognosis and response to anthracyclines. Technical issues, the cutoff levels used to classify a gene as amplified or deleted and different quality assurance guidelines could also explain these discrepancies [21,22].

Nevertheless, the molecular variations within the Chr17q amplicon and their clinical implications remain largely unknown. Indeed, besides TOP2A, other genes which are located not only in the HER2 smallest region of amplification (SRA) [23,24] but also all along the long arm of the Chr17 [5,24-27] might also be amplified, and they could play functional roles in breast cancer development and progression. To explore this hypothesis, we carried out a quantitative analysis of a set of 11 genes that were chosen for their localization along Chr17q12-q21 and for their putative role in breast cancer development. Two genes were located on the centromeric side (MED1 and STARD3), and nine were located on the distal side (GRB7, THRA, RARA, TOP2A, IGFBP4, CCR7, KRT20, KRT19 and GAS) of Chr17q12-21 relative to HER2 localization. The relation between gene amplification and patients’ characteristics and survival was then analyzed. We show that amplification of TOP2A and of other genes distal to HER2 does not occur in tumors in which HER2 is not amplified. We confirm that TOP2A is not included in the SRA involving HER2 and describe different copy number alterations along the distal side of Chr17q. We also show that TOP2A nonamplification is related to worse survival in the subgroup of patients with HER2-positive tumors.

Materials and methods

Patients’ characteristics, tumor samples and treatment

A total of 759 consecutive breast cancer patients referred to the Val d’Aurelle Cancer Center between March 2002 and May 2005 were prospectively entered into the database of a tumor DNA bank. Ethical approval was provided by the local research ethics committee. All patients gave their written, informed consent. The DNA bank was created using frozen, histologically proven invasive breast cancer specimens that were primarily handled for ER testing by the dextran charcoal method. Patients with primary HER2-negative breast cancer were initially selected on the basis of the level of HER2 expression by immunohistochemistry (IHC) using the HercepTest (DakoCytomation, Trappes, France). HER2 scores of 0 and 1 were considered negative. HER2 scores of 2+ were then checked by FISH for HER2 copy number (PathVysion HER-2 DNA Probe Kit; Abbott Molecular, Rungis, France). Only patients with HER2 scores of 3+ derived by IHC or positive for HER2 amplification by FISH were considered HER2-positive. HER2 copy number and Chr17 polysomy were then assessed by quantitative polymerase chain reaction (qPCR) as previously described [28]. Patients presenting with Chr17 polysomy without true HER2 amplification were excluded from the study. Adjuvant treatment was discussed in multidisciplinary medical meetings according to local and international guidelines and submitted for each patient’s approval. All patients were treated at least by adjuvant radiation therapy and received anthracycline-based adjuvant therapy according to the medical standards at the time of diagnosis. Considering the patient accrual period, and despite their HER2-positive status, women were not treated with adjuvant anti-HER2 therapy, with

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the exception of patients included in a clinical trial to assess the benefits of trastuzumab as adjuvant therapy. The clinicopathologic characteristics and treatments are summarized in Table 1. Patients received regular follow-up examinations, that is, every 6 months for the first 5 years, then yearly after that.

**DNA extraction and gene quantification**

Each frozen tumor specimen was pulverized in liquid nitrogen with an automatic grinder (Cryobroyeur 2000P Automatique; Rivoire, Montpellier, France), homogenized in a Polytron homogenizer in buffer (20 mM Tris HCl, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Na₂MoO₄, 1.5 mM dithiothreitol and 10% glycerol, pH 7.4) (buffer:tissue ratio was 10:1 (vol/wt)) used for ER and PR measurement and centrifuged at 10,000 x g for 15 minutes. Total genomic DNA was extracted from the pellet. The DNA concentration was determined by measuring the absorbance at 260 nm, and all samples had a 260/280-nm ratio higher than 1.7. DNA was stored at -20°C in TE buffer (10 mM Tris and 0.5 mM EDTA, pH 7.6). All procedures were carried out at 4°C unless noted otherwise. qPCR amplifications were performed with 10 ng of DNA on a Rotorgene 6000 apparatus (Qiagen, Courtaboeuf, France) using ABsolute Blue qPCR SYBR Green Mix (ref AB-4167; ThermoFisher Scientific, Illkirch, France) with an initial 15-minute denaturation step at 95°C followed by 40 cycles. The cycling temperatures and the primers used for each gene are summarized in Table 2. After completion of the amplification, samples were subjected to a temperature ramp (from the annealing temperature to 95°C with a transition rate of 0.1°C/s) with continuous fluorescence monitoring for melting curve analysis.

| MED1, STARD3, HER2, GRB7, THRA, RARA, TOP2A, IGFBP4, CCR7, KRT20, KRT19, GAS | MED1, STARD3, HER2, GRB7, THRA, RARA, TOP2A, IGFBP4, CCR7, KRT20, KRT19, GAS |
|-----------------------------------------------|-----------------------------------------------|
| **Table 1 Patients’ characteristics**        | **Table 1 Patients’ characteristics**        |
| Characteristic                             | Number of patients (%)                       |
|-----------------------------------------------|-----------------------------------------------|
| **Age, yr**                                 |                                               |
| <50                                          | 33 (38.4%)                                     |
| ≥50                                          | 53 (61.6%)                                     |
| **T**                                       |                                               |
| 1                                            | 35 (40.7%)                                     |
| 2                                            | 46 (53.5%)                                     |
| 3                                            | 4 (4.6%)                                       |
| 4                                            | 1 (1.2%)                                       |
| **N**                                       |                                               |
| N+                                           | 51 (59.3%)                                     |
| N                                           | 35 (40.7%)                                     |
| **SBR**                                     |                                               |
| 2                                            | 36 (42.3%)                                     |
| 3                                            | 49 (57.7%)                                     |
| Missing                                     | 1                                             |
| **Hormone receptor status**                  |                                               |
| ER                                           |                                               |
| ER                                          | 60 (69.8%)                                     |
| ER*                                         | 26 (30.2%)                                     |
| PR                                          |                                               |
| PR                                          | 44 (51.2%)                                     |
| PR*                                         | 42 (48.8%)                                     |
| ER/PR                                       |                                               |
| ER/PR                                       | 42 (48.9%)                                     |
| ER/PR*                                      | 18 (20.9%)                                     |
| ER*/PR                                      | 2 (2.3%)                                       |
| ER/*PR                                      | 24 (27.9%)                                     |
| **Treatment**                               |                                               |
| Chemotherapy                                |                                               |
| Anthracyclines                              | 42 (48.8%)                                     |
| Anthracyclines and taxanes                  | 26 (30.2%)                                     |
| No chemotherapy                             | 21 (24.0%)                                     |
| Radiation therapy                           | 86 (100.0%)                                    |
| Antihormone therapy                         | 34 (39.5%)                                     |
| Trastuzumab                                  | 20 (23.3%)                                     |
| **Outcomes**                                |                                               |
| Recurrence                                  | 25 (29.1%)                                     |
| Death                                       | 17 (20.0%)                                     |

*1, tumor size; N, lymph node status; SBR, Scarff-Bloom-Richardson histological grade; ER, estrogen receptor; PR, progesterone receptor.

**Statistical analysis**

Categorical variables were described using frequency distributions, and continuous variables were stated using medians and ranges. Correlations between genes was evaluated using the Spearman’s ρ correlation. Association between categorical variables was assessed with the χ² test (or Fisher’s exact test when appropriate). Differences were considered statistically significant when
Survival rates were estimated from the date of surgery to the date of the event of interest using the Kaplan-Meier method. The median survival was calculated with 95% confidence interval (95% CI). For overall survival (OS), the event of interest was death, regardless of the cause. Patients lost to follow-up were censored at the last documented visit. For relapse-free survival (RFS), the event of interest was recurrence. Patients who were alive at the last follow-up examination and without recurrence were censored at the time of the last follow-up examination. Patients who died without recurrence were censored at the date of death. Differences in survival rates were compared using a log-rank test. Statistical analyses were performed with Stata 10.0 software (Stata Corp., College Station, TX, USA).

### Results

#### Description of the study population

Eighty-six patients with HER2-amplified breast cancer were included in the study. The median follow-up period was 55 months (range, 6 to 81 months). A total of 25 patients (29%) relapsed after a median of 18 months from the date of surgery (range, 2 to 71 months), and 17 patients (20%) died after a median of 31 months (range, 6 to 78 months). The 5-year RFS rate was 69% (95% CI, 57% to 78%) and the 5-year OS rate was 79% (95% CI, 67% to 87%). There were no statistical associations between RFS or OS and the adjuvant treatment administered. The distribution of tumors relative to their pattern of amplified and nonamplified genes was homogeneous in the different treatment groups.

#### Table 2 Primer sequences and qPCR conditions used to quantify MED1, STARD3, HER2, GRB7, THRA, RARA, TOP2A, IGFBP4, CCR7, KRT20, KRT19, GAS, ACBT, GAPDH and SSTR2

| GenBank accession number | Gene name/primer | Sequence 5’-3’ | Size nt | GC, % | Amplicon, bp | Annealing temperature |
|--------------------------|------------------|----------------|---------|-------|--------------|----------------------|
| NP_004765                | MED1 sense       | ATT CTC CTG GGC TTC TCC AA | 20      | 50%   | 97           | 54°C                 |
| NP_006795                | MED1 antisense   | CCA CAC ACC AGG GAG TCA TT | 20      | 55%   | 97           | 54°C                 |
| NP_004795                | STARD3 sense     | CAG GCT GCT AGG GTG TAA CTG | 21      | 57%   | 114          | 64°C                 |
| NP_004795                | STARD3 antisense | GAC AGA GCA CCG GAG AAC AG | 20      | 60%   | 97           | 54°C                 |
| NM_004448                | HER2 sense       | GTC CCC CAT ATG TCT CCC G | 19      | 63%   | 101          | 58°C                 |
| NM_004448                | HER2 antisense   | CCG GAC ATG GTC TAA GAG GC | 20      | 60%   | 97           | 54°C                 |
| NP_005301                | GRB7 sense       | CTC TGG CTC AGA ACT TCC TGA AT | 23      | 48%   | 142          | 60°C                 |
| NP_005301                | GRB7 antisense   | GTC CCC TGG GTG GAG TAA TAG AG | 23      | 52%   | 97           | 54°C                 |
| NP_003241                | THRA sense       | GTG GAC AAG ATC GAG AGT CAG | 24      | 50%   | 119          | 60°C                 |
| NP_003241                | THRA antisense   | AGG TCA GTC ACC TTC ATC AGC AG | 23      | 52%   | 97           | 54°C                 |
| NP_000055                | RARA sense       | GGA GTG CTC AGA GTG GGT TC | 22      | 55%   | 114          | 64°C                 |
| NP_000055                | RARA antisense   | AGA AGG TCA TGG TGT CCT GCT C | 23      | 43%   | 121          | 58°C                 |
| NP_001058                | TOP2A sense      | GAT TCT GGA CCA ACC TTC AAC TA | 28      | 36%   | 114          | 60°C                 |
| NP_001058                | TOP2A antisense  | ATG TAC CAT CCT ACT AAC AAC TCT T | 28      | 36%   | 114          | 60°C                 |
| NP_001543                | IGFBP4 sense     | CTC TGG CTC AGA ACT TCC TGA CT | 20      | 60%   | 146          | 60°C                 |
| NP_001543                | IGFBP4 antisense | GGT GCT CCG GTC TCG AAT | 18      | 61%   | 97           | 54°C                 |
| XP_00267                 | CCR7 sense       | AGG TCA AAT CCC AGC CAG AG | 20      | 55%   | 127          | 58°C                 |
| XP_00267                 | CCR7 antisense   | CTC TGG TGT TGT CTC CGA TG | 20      | 55%   | 127          | 58°C                 |
| NP_001920                | KRT20 sense      | ATG GCT TCA GAG GGA CCA GGT | 21      | 48%   | 95           | 55°C                 |
| NP_001920                | KRT20 antisense  | TGG AGA TCA TGG TCT TCC ACT GTA | 22      | 45%   | 97           | 54°C                 |
| NP_002267                | KRT19 sense      | TGA CAT GCG AAG CCA ATA TG | 20      | 45%   | 124          | 56°C                 |
| NP_002267                | KRT19 antisense  | AAA GCC CTC CCC TCC TCA AC | 20      | 55%   | 97           | 54°C                 |
| XP_00183                 | GAS sense        | TCT CCC GAG ACT GGC TCT GA | 20      | 60%   | 146          | 64°C                 |
| XP_00183                 | GAS antisense    | GCC GAA GTC CAT CCA TCC AT | 20      | 55%   | 97           | 54°C                 |
| NP_001041                | SSTR2 sense      | GGC TCC AGG GTC CAT TAA GG | 20      | 60%   | 101          | 60°C                 |
| NP_001041                | SSTR2 antisense  | ATT GAG TCT GTC TCC CGC C | 19      | 58%   | 97           | 54°C                 |
| NP_001092                | ACBT sense       | CCA CAC TGT GCC CAT CTA CG | 20      | 60%   | 99           | 65°C                 |
| NP_001092                | ACBT antisense   | AGG ATC TCC ATG AGG TAG TCA GTC AG | 26      | 46%   | 97           | 54°C                 |
| NT_000759                | GAPDH sense      | CTC ACG TAT TCC CCC AGG TT | 20      | 55%   | 161          | 58°C                 |
| NT_000759                | GAPDH antisense  | CCC AAA GCA CAT TCC TTC CA | 20      | 45%   | 97           | 54°C                 |

qPCR, quantitative polymerase chain reaction; MED1, mediator complex subunit 1 gene; STARD3, steroidogenic acute regulatory-related lipid transfer (START) domain containing 3 gene; HER2, human epidermal growth factor receptor 2 gene; GRB7, growth factor receptor-bound protein 7 gene; THRA, thyroid hormone receptor a gene; RARA, retinoic acid receptor a gene; TOP2A, topoisomerase IIa gene; IGFBP4, insulin-like growth factor-binding protein 4 gene; CCR7, C-C chemokine receptor type 7 gene; KRT20, cytokeratin 20 gene; KRT19, cytokeratin 19 gene; GAS, gastrin gene; ACBT, b-actin gene; GAPDH, glyceraldehyde 3-phosphate dehydrogenase gene; SSTR2, somatostatin receptor type II gene.

P < 0.05. Survival rates were estimated from the date of surgery to the date of the event of interest using the Kaplan-Meier method. The median survival was calculated with 95% confidence interval (95% CI). For overall survival (OS), the event of interest was death, regardless of the cause. Patients lost to follow-up were censored at the last documented visit. For relapse-free survival (RFS), the event of interest was recurrence. Patients who were alive at the last follow-up examination and without recurrence were censored at the time of the last follow-up examination. Patients who died without recurrence were censored at the date of death. Differences in survival rates were compared using a log-rank test. Statistical analyses were performed with Stata 10.0 software (Stata Corp., College Station, TX, USA).
Analysis of the Chr17q12-q21 amplicon by qPCR
The variability in the efficiency of each parameter was between 92% and 105% with a standard deviation of 0.04. The reference genes (SSTR2, GAPDH and ACBT) were chosen in regions previously described as presenting a low level of instability or amplification [25,26] and were controlled together to detect amplification. Their crossing point values were 19.2 ± 0.56, 18.7 ± 0.47 and 17.6 ± 0.43, respectively. The absence of SSTR2 amplification was confirmed in all samples by measuring the SSTR2:GAPDH and SSTR2:ACBT ratios, which in all cases were <1.5. Finally, none of the 40 HER2-negative cancer specimens (negative controls) showed amplification of the studied genes. The status (amplified, deleted or normal) of the 11 genes (MED1, STARD3, GRB7, THRA, RARA, TOP2A, IGFBP4, CCR7, KRT20, KRT19 and GAS) evaluated in the 86 HER2-positive cancer samples is summarized in Table 3. Coamplification with HER2 occurred in 65.1% of samples for MED1 (56 of 86), 93% for STARD3 (80 of 86), 97.7% for GRB7 (84 of 86), 54.7% for THRA (47 of 86), 26.7% for RARA (23 of 86), 26.7% for TOP2A (23 of 86), 20.9% for IGFBP4 (18 of 86), 20.9% for CCR7 (18 of 86), 14% for KRT20 (12 of 86), 11.6% for KRT19 (10 of 86) and 7% for GAS (6 of 86). HER2 presented the highest level of amplification (mean, 6.4; range, 2 to 32), whereas the frequency of amplification of the other genes decreased according to the distance of the gene from HER2 (Figure 1). The amplification levels of HER2 and of the other genes were compared by linear regression analysis. HER2 amplification levels were correlated only with the amplification levels of MED1 (r = 0.84), STARD3 (r = 0.87) and GRB7 (r = 0.93), but not of other genes, particularly TOP2A (r = 0.65). Gene deletions occurred in fewer than 5.8% of the samples, regardless of the gene studied (Table 3).

Relation between the pattern of gene amplification and clinical characteristics
Node status (N), Scarff-Bloom-Richardson histological (SBR) grade and ER/PR-negative status were significantly associated with worse outcomes in terms of both RFS rate (P = 0.007, P = 0.004 and P = 0.024, respectively) and OS rate (P = 0.020, P = 0.048 and P = 0.076, respectively) (univariate analysis) (see Table 4 and Figure 2 for ER/PR status). The patterns of gene amplification were not significantly correlated with the T or SBR grade. Conversely, MED1 amplification tended to be associated with ER-positive tumors (P = 0.072), whereas amplification of RARA, KRT20 or KRT19 was significantly associated with node-positive breast cancers (P = 0.030, P = 0.002 and P = 0.033, respectively). Moreover, patients with TOP2A-amplified tumors tended to have better RFS rates (hazard ratio (HR) = 0.47, 95% CI

| Gene     | Number of patients (%) | Median (range) |
|----------|------------------------|----------------|
| SSTR2    | Deletion 3 (3.5%)       | 0.5 (0.5 to 0.5) |
|          | Normal 27 (31.4%)       | 0.9 (0.6 to 1.9) |
|          | Amplification 56 (65.1%) | 4.0 (2 to 27.8)  |
|          | Total 86 (100.0%)       | 2.6 (0.5 to 27.8) |
| STARD3   | Deletion 0              | -              |
|          | Normal 6 (7.0%)         | 1.5 (0.7 to 1.9) |
|          | Amplification 80 (93.0%) | 6.4 (2.0 to 74.9) |
|          | Total 86 (100.0%)       | 5.9 (0.7 to 74.9) |
| HER2     | Amplification 86 (100.0%) | 6.4 (2 to 32)   |
| GRB7     | Deletion 0              | -              |
|          | Normal 2 (2.3%)         | 1.1 (0.6 to 1.7) |
|          | Amplification 84 (97.7%) | 6.8 (2.3 to 36.5) |
|          | Total 86 (100.0%)       | 6.6 (0.6 to 36.5) |
| THRA     | Deletion 1 (1.2%)       | 0.5             |
|          | Normal 38 (44.2%)       | 1.1 (0.6 to 1.9) |
|          | Amplification 47 (54.7%) | 4.4 (2.0 to 11.7) |
|          | Total 86 (100.0%)       | 2.2 (0.5 to 11.7) |
| RARA     | Deletion 3 (3.5%)       | 0.5 (0.4 to 0.5) |
|          | Normal 60 (69.8%)       | 1.0 (0.6 to 1.9) |
|          | Amplification 23 (26.7%) | 3.4 (2.0 to 11.1) |
|          | Total 86 (100.0%)       | 1.1 (0.4 to 11.1) |
| TOP2A    | Deletion 4 (4.7%)       | 0.5 (0.5 to 0.5) |
|          | Normal 59 (68.6%)       | 1.0 (0.6 to 1.8) |
|          | Amplification 23 (26.7%) | 3.2 (2.7 to 32)  |
|          | Total 86 (100.0%)       | 1.1 (0.5 to 7)   |
| IGFBP4   | Deletion 5 (5.8%)       | 0.5 (0.5 to 0.5) |
|          | Normal 63 (73.3%)       | 1.0 (0.6 to 1.9) |
|          | Amplification 18 (20.9%) | 3.1 (2 to 8)     |
|          | Total 86 (100.0%)       | 1.1 (0.5 to 8)   |
| CCR7     | Deletion 3 (3.5%)       | 0.4 (0.4 to 0.5) |
|          | Normal 65 (75.6%)       | 0.9 (0.6 to 1.8) |
|          | Amplification 18 (20.9%) | 3.3 (2.0 to 14.9) |
|          | Total 86 (100.0%)       | 1.0 (0.4 to 14.9) |
| KRT20    | Deletion 2 (2.3%)       | 0.5 (0.5 to 0.5) |
|          | Normal 72 (83.7%)       | 1.1 (0.6 to 1.8) |
|          | Amplification 12 (14.0%) | 3.1 (2.3 to 11.6) |
|          | Total 86 (100.0%)       | 1.1 (0.5 to 11.6) |
| KRT19    | Deletion 5 (5.8%)       | 0.5 (0.5 to 0.5) |
|          | Normal 71 (82.6%)       | 1.0 (0.6 to 1.8) |
0.16 to 1.38, \(P = 0.159\) and OS rates (HR = 0.34, 95% CI 0.08 to 1.48, \(P = 0.132\)) than patients with TOP2A nonamplified tumors (Figure 2). The subgroup of patients with ER/PR-negative cancers without amplification of TOP2A in particular had the worst RFS rates (HR = 0.29, 95% CI 0.13 to 0.65, \(P = 0.001\)) and the worst OS rates (HR = 0.28, 95% CI 0.10 to 0.76, \(P = 0.008\)).

Discussion
In this work, we quantified gene amplification in the 17q12 to 17q21 region around HER2 in HER2-amplified breast tumors. Among the patients with primary breast cancer, fewer than 12% had HER2-positive tumors. This result is in accordance with recent studies reporting 12% to 15% of HER2 amplification compared to the 20% to 25% found in historical studies [29,30], in which Chr17
Table 4 Univariate analysis of the different variables

| Variable | Number of patients | Events | 5-year RFS | HR (95% CI) | Events | 5-year OS | HR (95% CI) |
|----------|--------------------|--------|------------|-------------|--------|-----------|-------------|
| T        |                    |        |            |             |        |           |             |
| 1        | 35                 | 8      | 74.2       | 1           | 6      | 84.2      | 1           |
| 2        | 46                 | 15     | 65.9       | 1.51 (0.64 to 3.58) | 10     | 74.3      | 1.30 (0.47 to 3.60) |
| 3 or 4   | 5                  | 2      | 53.3       | 2.15 (0.45 to 10.4) | 1      | 80.0      | 1.34 (0.16 to 11.3) |
| N        |                    |        |            |             |        |           |             |
| N⁺       | 35                 | 4      | 88.1       | 1           | 2      | 94.2      | 1           |
| N⁻       | 51                 | 21     | 55.8       | 3.94 (1.35 to 11.3) | 15     | 70.0      | 4.89 (1.12 to 21.4) |
| P        | 0.510*             |        |            |             | 0.007* |           |             |
| SBR      |                    |        |            |             |        |           |             |
| 2        | 36                 | 5      | 84.9       | 1           | 4      | 89.4      | 1           |
| 3        | 49                 | 20     | 55.8       | 3.80 (1.42 to 10.2) | 13     | 70.8      | 2.96 (0.96 to 9.15) |
| ER       |                    |        |            |             |        |           |             |
| ER⁺      | 60                 | 19     | 66.9       | 1           | 13     | 76.2      | 1           |
| ER⁻      | 26                 | 6      | 70.1       | 0.64 (0.25 to 1.60) | 4      | 84.9      | 0.68 (0.22 to 2.10) |
| P        | 0.333*             |        |            |             | 0.004* |           |             |
| PR       |                    |        |            |             |        |           |             |
| PR⁺      | 44                 | 18     | 57.6       | 1           | 13     | 67.3      | 1           |
| PR⁻      | 42                 | 7      | 80.9       | 0.36 (0.15 to 0.87) | 4      | 92.4      | 0.32 (0.10 to 0.99) |
| P        | 0.018*             |        |            |             | 0.004* |           |             |
| ER/PR    |                    |        |            |             |        |           |             |
| ER⁻/PR⁻  | 42                 | 17     | 58.7       | 1           | 12     | 68.9      | 1           |
| Other    | 44                 | 8      | 78.2       | 0.39 (0.17 to 0.91) | 5      | 89.2      | 0.40 (0.14 to 1.14) |
| P        | 0.024*             |        |            |             | 0.024* |           |             |
| MED1     |                    |        |            |             |        |           |             |
| Del/Nm   | 30                 | 10     | 66.2       | 1           | 7      | 72.9      | 1           |
| Amp      | 56                 | 15     | 70.0       | 0.76 (0.34 to 1.70) | 10     | 82.5      | 0.63 (0.23 to 1.69) |
| P        | 0.507*             |        |            |             | 0.354* |           |             |
| STARD3   |                    |        |            |             |        |           |             |
| Del/Nm   | 6                  | 2      | 55.6       | 1           | 2      | 53.3      | 1           |
| Amp      | 80                 | 23     | 69.4       | 0.85 (0.20 to 3.62) | 15     | 80.9      | 0.48 (0.11 to 2.14) |
| P        | 0.826*             |        |            |             | 0.327* |           |             |
| GRB7     |                    |        |            |             |        |           |             |
| Del/Nm   | 2                  | 1      | 50.0       | 1           | 1      | 0.00      | 1           |
| Amp      | 84                 | 24     | 69.3       | 0.30 (0.04 to 2.33) | 16     | 80.1      | 0.15 (0.02 to 1.20) |
| P        | 0.225*             |        |            |             | 0.038* |           |             |
| THRA     |                    |        |            |             |        |           |             |
| Del/Nm   | 39                 | 16     | 59.0       | 1           | 11     | 73.8      | 1           |
| Amp      | 47                 | 9      | 79.3       | 0.50 (0.22 to 1.13) | 6      | 94.7      | 0.53 (0.19 to 1.43) |
| P        | 0.090*             |        |            |             | 0.199* |           |             |
| RARA     |                    |        |            |             |        |           |             |
| Del/Nm   | 63                 | 21     | 64.1       | 1           | 15     | 75.2      | 1           |
| Amp      | 23                 | 4      | 82.4       | 0.50 (0.17 to 1.45) | 2      | 91.3      | 0.36 (0.08 to 1.59) |
| P        | 0.190*             |        |            |             | 0.162* |           |             |
| TOP2A    |                    |        |            |             |        |           |             |
| Del/Nm   | 63                 | 21     | 63.7       | 1           | 15     | 74.8      | 1           |
| Amp      | 23                 | 4      | 82.4       | 0.47 (0.16 to 1.38) | 2      | 91.3      | 0.34 (0.08 to 1.48) |
| P        | 0.159*             |        |            |             | 0.132* |           |             |
polysomy may have led to discordant interpretations between higher signals due to polysomy and those due to an absolute increase of HER2 gene copy number in a significant proportion of cases [31]. Gene amplification on the centromeric side (MED1 and STARD3) and the distal side (GRB7, THRA, RARA, TOP2A, IGFBP4, CCR7, KRT20, KRT19 and GAS) relative to HER2 localization on Chr17q was determined by qPCR, an efficient method for performing copy number analysis [32], and with reference genes located on Chr17 (STARD2) and Chr4 and Chr2 (GAPDH and ACBT, respectively) to differentiate polysomy from true amplification. In fact, tumors presenting only Chr17 polysomy should not be considered as HER2-amplified, and patients with this presentation might not benefit from anti-HER2 therapy, although these tumors present moderate HER2 overexpression [28,33], but only as a result of treatment with anthracyclines [34].

We have confirmed that gene amplification, including that of TOP2A, occurs only in patients with HER2-amplified breast tumors. We then showed that HER2 presents the highest amplification value and that the frequency of amplification of a gene decreases with its distance from HER2. These results suggest that instability within Chr17q is driven by HER2 amplification. Indeed, HER2-positive tumors have higher levels of overall genomic instability than HER2-negative tumors, supporting the idea that HER2 amplification is the key amplicon driver on Chr17q [35,36]. Moreover, we found a correlation between the amplification levels of HER2 and that of MED1, STARD3 and GRB7, but not of TOP2A and the other genes localized on the distal side of Chr17q. Therefore, the SRA of HER2 seems to be limited to a small number of genes, among which are STARD3 and GRB7 as described by Katoh et al. [37] and as recently reported by Beroukhim et al. [38] in different cancer types. Kuwahara et al. [39] identified a region between HER2 and GRB7 (a gene located 47 kb from HER2) that contains a recombinant hot spot for amplification, deletion and translocation. This site might be located between GRB7 and THRA, a gene located 315 kb from GRB7, given that 84 (97.7%) of the cancer specimens we assessed presented GRB7 amplification, whereas only 47 (54.7%) showed THRA amplification. The presence of this hot spot might explain why the
**Figure 2** Kaplan-Meier curves for relapse-free survival (RFS) and overall survival (OS). (a) Estrogen receptor/progesterone receptor (ER/PR) status. Blue line indicates ER- and PR-negative (ER-/PR-) status; black line indicates ER-, PR- or ER/PR-positive (ER-/PR+, ER+/PR- or ER+/PR+) status. (b) Topoisomerase IIα (TOP2A) amplification. Blue line indicates deleted or nonamplified (normal) TOP2A (TOP2A Del/N); black line indicates amplified TOP2A (TOP2A Amp). For RFS, $P = 0.159$; for OS, $P = 0.132$. (c) Top2A and ER/PR status. Blue line indicates ER- and PR-negative (ER-/PR-) and deleted or nonamplified TOP2A (TOP2A Del/N); black line indicates ER-, PR-, or ER/PR-positive (ER-/PR+, ER+/PR- and ER+/PR+) status and amplified TOP2A (TOP2A Amp). For RFS, $P = 0.001$; for OS, $P = 0.008$. 

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frequency of recombination for a gene is higher when it is located closer to HER2 and gradually decreases for genes located far away from HER2. In general, the gene amplification architectures derived from our results illustrate the complexity of the rearrangement mechanisms (sister chromatid breakage-fusion-bridge cycles, formation and reinsertion of double minutes and repeated units at a single locus) and highlight the diversity of HER2-amplified cancers [40,41].

Finally, we found a low overall number of deletions (<5.8%), regardless of the gene analyzed. For TOP2A, the deletion rate was 4.7%, which is low compared with values found in other studies (between 8.1% and 35%) [22]. This might be explained by the fact that in heterogenic tumors, which simultaneously present amplifications and deletions, qPCR cannot detect deletions that are present in a very small number of cells. It is also possible that in certain studies the percentage of tumors with deletions was overestimated as a result of poor standardization of the FISH technique. Indeed, Di Leo et al. [42,43] recently showed high interlaboratory variations for TOP2A quantification, whereas Press et al. [44] found a deletion rate comparable to ours in a prospective study using standardized FISH.

We then investigated the clinical impact of Chr17 anomalies. Amplification of RARA, KRT20 and KRT19 correlated with node invasion, suggesting that tumors with amplification of genes on the distal side of Chr17q are more aggressive than tumors with amplifications restricted to the SRA of HER2. MED1 amplification showed a tendency to be associated with ER-positive tumors. MED1 encodes the mediator complex subunit 1 (MED1) [45] that anchors mediator to ERβ [46] while also interacting with ERS [47]. Although our study did not show any significant impact of MED1 amplification on survival, the prognostic value of MED1 should nevertheless be evaluated in a larger ER/PR-positive population. Similarly, TOP2A amplification was not a statistically significant prognostic factor, but patients with tumors in which TOP2A was amplified tended to have better RFS and OS rates (see Figure 2b). This result is in accord with a recent study in which patients with HER2-positive, TOP2A-amplified cancers presented a trend of better survival than patients with HER2-positive breast cancers with deleted or normal TOP2A [48]. Conversely, the subgroup of patients with ER/PR-negative breast cancers and nonamplified (normal or deleted) TOP2A showed the least favorable RFS and OS rates in our population. We could not determine whether the prognosis was even less favorable in the case of ER/PR-negative tumors with TOP2A-deleted status than of tumors with normal TOP2A. Nevertheless, TOP2A deletions, which are often heterozygous [49], do not result in total loss of expression of the protein [50]. Their unfavorable prognosis might be explained by the fact that ER/PR-negative, TOP2A nonamplified tumors may not be responsive to hormone therapy and/or to anthracycline-based therapy because of the absence or insufficient number of targets. Adjuvant therapy would thus not be beneficial for these patients. Indeed, recent findings suggest that the presence of TOP2A deletions is not associated with better chemosensitivity or response to anthracyclines, while the occurrence of TOP2A amplifications may identify a subgroup of patients with increased response to anthracyclines [18,44]. Many studies have been conducted on the impact of the presence of HER2 and TOP2A amplifications on survival and response to chemotherapy. Only a few studies found that there was no benefit to treating patients with HER2-positive cancer with anthracyclines or that TOP2A amplification had no predictive value. The subject remains controversial [51], but nevertheless two large trials (the National Surgical Adjuvant Breast and Bowel Project B-11 and the National Cancer Institute of Canada MA.5 studies) have retrospectively shown a correlation between HER2 amplification and benefit from adjuvant anthracycline-based chemotherapy [52,53]. Moreover, a pooled analysis that included 8 studies and 1,536 HER2-positive patients indicated that anthracyclines were superior to non-anthracycline-based regimens in terms of disease-free and overall survival [54].

Conclusions

Few studies have used qPCR to assess gene copy number alterations in HER2-amplified breast cancer. The assay we have developed can be used to assess the amplification status of multiple genes. We have shown that MED1, STARD3, TOP2A, GRB7, THRA, RARA, IGFBP4, CCR7, KRT19, KRT20 and GAS amplifications may identify a subgroup of patients with HER2-amplified breast cancers with variable frequencies. We have confirmed that HER2 seems to drive genomic instability along Chr17q and that TOP2A was not included in the SRA of HER2. The different patterns of amplification we observed suggest complex amplification mechanisms leading to a variety of subtypes. RARA, KRT19 and KRT20 amplification could be related to a more invasive breast cancer profile. Moreover, we confirm the clinical importance of identifying the subgroup of patients with ER/PR-negative, TOP2A nonamplified cancer, as they have the worst prognosis. A prospective study including a larger number of patients treated with trastuzumab and anthracycline-based adjuvant therapy would be useful to determine the predictive value of TOP2A amplification and the prognostic value of gene copy number variations along Chr17q.
Abbreviations
ACBT: β-actin; CCR7: C-C chemokine receptor type 7; Chr17q: chromosome 17q; ER: estrogen receptor; FISH: fluorescence in situ hybridization; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GAS: gastrin; GRB7: growth factor receptor-bound protein 7; HER2: human epidermal growth factor receptor 2; IGFBP4: insulin-like growth factor-binding protein 4; KRT20: cytokeratin 20; LAMMY: Lamy receptor-bound protein 7; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GAS: gastrin; GRB7: growth factor receptor-bound protein 7; HER2: human epidermal growth factor receptor 2; IGFBP4: insulin-like growth factor-binding protein 4; KRT20: cytokeratin 20; MED1: mediator complex subunit 1; N: nodal status; OS: overall survival; PR: progesterone receptor; qPCR: quantitative polymerase chain reaction; RARA: retinoic acid receptor; SBR grade: Scarff-Bloom-Richardson histological grade; SRA: small region of amplification; SSTR2: somatostatin receptor type II; STARD3: steroidogenic acute regulatory-related lipid transfer (START) domain containing 3; TRHR: thyrotrphin-releasing hormone receptor α; TOP2A: topoisomerase IIα; T: tumor size.

Acknowledgements
This work was funded by the Assistance Publique des Hôpitaux de Marseille (APHM) and the Val d’Aurelle Cancer Center. We thank Philippe Rouanet and Gilles Romieu for clinical samples. We are grateful to E. Andermacher for editing the manuscript.

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Authors’ contributions
PJL contributed to the conception and design of the entire study, coordinated sample collection, interpreted data and drafted the manuscript. FF made the first design of the quantitative polymerase chain reaction (qPCR) assay, contributed to data interpretation as well as statistical analysis and assisted in drafting the manuscript. CMB supervised the statistical analysis and assisted in drafting the manuscript. ACL was responsible for designing and optimizing the qPCR assays and carried out most of the experiments with the DNA samples described in this paper. PMM and LO made original observations leading to this work and contributed to the critical revision of the manuscript. WJ selected the eligible patients, acquired the clinicopathological data and contributed to the drafting of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 28 September 2010 Revised: 19 December 2010 Accepted: 2 February 2011 Published: 2 February 2011

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Cite this article as: Lamy et al.: Quantification and clinical relevance of gene amplification at chromosome 17q12-21 in human epidemic growth factor receptor 2-amplified breast cancers. Breast Cancer Research 2011 13:R15.