Tumor-Initiating Cells of HER2-Positive Carcinoma Cell Lines Express the Highest Oncoprotein Levels and Are Sensitive to Trastuzumab

Alessandra Magnifico,1 Luisa Albano,1 Stefano Campaner,4 Domenico Delia,2 Fabio Castiglioni,1 Patrizia Gasparini,3 Gabriella Sozzi,3 Enrico Fontanella,2 Sylvie Menard,1 and Elda Tagliabue1

Abstract

Purpose: The existence of tumor-initiating cells in breast cancer has profound implications for cancer therapy. In this study, we investigated the sensitivity of tumor-initiating cells isolated from human epidermal growth factor receptor type 2 (HER2)-overexpressing carcinoma cell lines to trastuzumab, a compound used for the targeted therapy of breast cancer.

Experimental Design: Spheres were analyzed by indirect immunofluorescence for HER2 cell surface expression and by real-time PCR for HER2 mRNA expression in the presence or absence of the Notch1 signaling inhibitor (GSI) or Notch1 small interfering RNA. Xenografts of HER2-overexpressing breast tumor cells were treated with trastuzumab or doxorubicin. The sphere-forming efficiency (SFE) and serial transplantability of tumors were assessed.

Results: In HER2-overexpressing carcinoma cell lines, cells with tumor-initiating cell properties presented increased HER2 levels compared with the bulk cell population without modification in HER2 gene amplification. HER2 levels were controlled by Notch1 signaling, as shown by the reduction of HER2 cell surface expression and lower SFE following γ-secretase inhibition or Notch1 specific silencing. We also show that trastuzumab was able to effectively target tumor-initiating cells of HER2-positive carcinoma cell lines, as indicated by the significant decrease in SFE and the loss of serial transplantability, following treatment of HER2-overexpressing xenografts.

Conclusions: Here, we provide evidence for the therapeutic efficacy of trastuzumab in debulking and in targeting tumor-initiating cells of HER2-overexpressing tumors. We also propose that Notch signaling regulates HER2 expression, thereby representing a critical survival pathway of tumor-initiating cells.

Increasing evidence indicates that a small subpopulation of tumor cells, called tumor-initiating cells, is not only responsible for the generation of the phenotypically diverse tumor cell populations, but also capable of long-term self-renewal, therefore supporting the growth and dissemination of cancers (1). Recent studies suggest that tumor-initiating cells might be particularly resilient to therapeutic approaches. For instance, breast tumor-initiating cells seem particularly radio-resistant due to more efficient DNA repair machinery (2), whereas chemotherapy has been reported to select for survival of tumor-initiating cells in breast carcinomas (3–5). Thus, it has become important to define the sensitivity of this critical subpopulation to current therapies.

About 20% of invasive breast carcinomas show overexpression of human epidermal growth factor receptor type 2 (HER2), and patients with HER2-positive tumors have a decreased overall survival rate (6–8). Recently, a humanized monoclonal antibody (mAb) against the extracellular domain of HER2, trastuzumab, has been approved by the Food and Drug Administration for treatment of patients with invasive HER2-overexpressing breast cancers (9). In this study, we characterized the tumor-initiating cells of HER2-positive carcinoma cell lines and explored the possible mechanism by which the tumor initiating cells of HER2-positive carcinoma cell lines present elevated HER2 expression compared with the bulk cell population. Furthermore, we investigated the sensitivity of tumor-initiating cells derived from HER2-positive carcinoma cell lines to trastuzumab by analyzing the spheres-forming efficiency (SFE) and transplantability of cells derived from tumor xenotransplants treated with this therapeutic agent.

Materials and Methods

Antibodies and reagents. The following antibodies were used for Western blotting analysis: anti-HER2 mAb Ab3 (1 μg/mL; Calbiochem); anti-phospho-tyrosine clone 4G10 mAb (1 μg/mL) and anti-phospho-EGFR (Tyr 1086) polyclonal antibody (0.1 μg/mL; Upstate Biotechnology); anti-EGFR 1005 polyclonal antibody (1 μg/mL), anti-EGFR 1005.
Tumor-initiating Cell Sensitivity to Trastuzumab

Evidence for the existence of tumor-initiating cells in breast cancer points to the likelihood that eradication of these cells is the critical determinant in achieving cure. Thus, changes in therapeutic modalities used in treating human epidermal growth factor receptor type 2 (HER2)-overexpressing tumors have highlighted the need to identify tumor-initiating cells and characterize their sensitivity to such a targeted therapy. In our study of HER2-overexpressing carcinoma cell lines, we provide evidence, for the first time, that trastuzumab can effectively target tumor-initiating cells. In light of the fact that the efficacy of trastuzumab depends on HER2 levels, we show that the results obtained are explained by the higher HER2 expression levels in tumor-initiating cells of HER2-positive tumors compared with the totality of cells forming the tumor. From a clinical perspective, we believe that our study provides a rationale of why and how current trastuzumab therapy exerts its action on HER2-positive breast carcinoma.

In vivo tumor growth. Six-week-old nude mice were injected in the mammary fat pad with $10^6$ MDA-MB-361, MCF7, or ZR-75-1 cells in low-attachment plates (Corning Inc.). Spheroid medium has been described (10). To exclude those spheres derived from cellular aggregates instead of division from a single cell, tumor cell lines were stained with PKH26 (red dye, ref. 11) or PKH67 (green dye, ref. 12) before seeding in nondifferentiating culture conditions. After 1 wk, individual spheres homogeneously stained with a single dye were considered clonally derived. Sphere-forming efficiency (SFE) was calculated as the number of spheres (diameter = 60 \mu m) divided by the original number of single cells seeded and expressed as a percentage (± SD; ref. 13). Differentiated cells were obtained from spheres seeded on matrigel for 14 d (14, 15) and tested for their tumorigenic capacity compared with spheres (10^6 versus 10^4).

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amount of DMSO for 6 h at 37°C. After washing with sphere medium, cells were plated in nondifferentiating conditions and analyzed for SFE 7 d later. Cells were analyzed for HER2 expression by indirect immunofluorescence, for HER2 mRNA expression by RT-PCR, and for Notch1 intracellular domain and Hes1 target gene expression by Western blotting.

**Silencing of Notch1 by siRNA transfection.** Cells seeded in 6-well low-attachment plates (10^3 cells/cm²) were transfected with either a small interfering RNA pool for human Notch1 (ON-TARGETplus SMARTpool, Human NOTCH1, final concentration 10-30 nmol/L; Dharmacon) or a pool of control RNA duplexes (On-TARGETplus Non-Targeting Pool, final concentration 10-30 nmol/L; Dharmacon) using 16 μL/well of INTERFERin transfection reagent (Polyplus transfection) into 1 mL of spheres medium. After overnight incubation at 37°C, spheres culture medium was added to a final volume of 2 mL/well. SFE were determined at 5 d posttransfection and spheres were tested for HER2 and Notch1 mRNA levels by real-time PCR. Notch1 and HER2 expression levels were determined by real-time PCR as above described using spheres silenced by a pool of control RNA duplexes as calibrator samples.

**In vitro drug treatment.** MDA-MB-361, BT-474, SKOV-3, ZR-75-1, and MCF7 cells treated or not in suspension with 25 to 50 to 100 μg/mL of trastuzumab or with 1 to 10 nmol/L of lapatinib were analyzed for both SFE efficiency and adhesion growth ability using MTT assay after 6 d of culture.

**Statistical analysis.** Data were compared using the Student’s t-test. Values were expressed as mean ± SD; differences were considered
Fig. 2. Tumor-initiating cell characteristics in cells expressing the highest HER2 surface levels. A, SFE analysis of sorted MDA-MB-361 HER2-Low and HER2-High cells (mean ± SD of six replicates). B, SFE of sorted SKOV-3 HER2-Low and HER2-High cells (mean ± SD of six replicates). C, Western blot analysis of whole cell lysates from HER2-Low and HER2-High MDA-MB-361 and SKOV-3 cells for expression of HER2, P-HER2, EGFR, P-EGFR, HER3, P-HER3, AKT, P-AKT, MAPK, P-MAPK, and stem cell markers Bmi1 and Oct4. D, representative fluorescence-activated cell sorting analysis of HER2 expression in MDA-MB-361 and SKOV-3 HER2-High spheres (grey area), HER2-High spheres grown on differentiating conditions (light grey line), and parental cells (dark grey line).
significant at $P \leq 0.05$. In the real-time PCR analyses, error bars represent the calculated maximum (RQMax) and minimum (RQMin) expression levels that represent 95% confidence interval of the mean expression level (RQ value).

Results

HER2 expression in tumor-initiating cells derived from HER2-positive carcinoma cell lines. An established approach to the isolation of tumor-initiating cells consists in growing cells in suspension under undifferentiating conditions (10). Under this condition, cells grow as undifferentiated spheroids structures (spheres) and tend to express characteristic stem cell markers such as Oct4 and Bmi1 (20–22). This method can be used to assess the self-renewal potential of both mammary gland progenitor cells and tumor-initiating cells.

We selected a panel of breast and ovarian cancer cell lines known to overexpress HER2 and bearing HER2 gene amplification, and compared the levels of HER2 surface expression of spheroid cultures with the corresponding “parental” cell line grown as monolayer or with cells derived from spheres that were differentiated by culturing in matrigel.

Analysis of HER2 cell surface expression in the breast carcinoma lines MDA-MB-361 and BT-474, or in the ovarian carcinoma line SKOV-3, revealed higher HER2 levels in spheres compared with the same cells induced to differentiate or the parental cell lines (2- to 7-fold increase; Fig. 1A). Similar results were obtained with the breast carcinoma cell line ZR-75-1, which overexpresses HER2 without gene amplification: HER2 expression in spheres was three times higher than in differentiated cells. No differences in HER2 levels were found in MCF7 cells, a cell line expressing low levels of HER2 (Fig. 1A).

We then analyzed HER2 gene copy number in tumor-initiating cells and in their differentiated counterparts by fluorescence in situ hybridization on the MDA-MB-361 cell line (Fig. 1B) and SKOV-3, BT-474, and ZR-75-1 cells (Supplementary Fig. S1). We found that the carcinoma cell lines with HER2 gene amplification showed similar ratio between HER2 gene copies and chromosome 17 centrosomes in tumor-initiating cells and differentiated cells. Similarly, the carcinoma cell line without gene amplification as ZR-75-1 cells (Supplementary Fig. S1C) maintained its HER2 gene property in tumor-initiating cells and differentiated counterparts. RT-PCR analysis indicated that spheres derived from MDA-MB-361, SKOV-3, and BT-474 and ZR-75-1 cells, expressed significantly more HER2 transcripts compared with cells grown under differentiating conditions (Fig. 1C). By contrast, MCF7 spheres showed similar levels of HER2 protein but lower HER2 transcripts levels compared with their counterparts cultured in matrigel.

Our data show that the high expression of HER2 in tumor-initiating cells is transcriptionally controlled and does not depend on increased gene dosage.

Given that previous studies have implicated the ALDH-positive population as possessing stem cell properties (23); we investigated the cell surface expression of HER2 in an ALDH-positive population by fluorescence-activated cell sorting.

Table 1. Tumor growth ability

A. Tumor growth of carcinoma HER2-High and HER2-Low cells at different dilutions

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HER2 levels</th>
<th>Outgrowths/injections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10^6 injected cells</td>
<td>10^5 injected cells</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>HER2-High</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>HER2-Low</td>
<td>6/6</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>HER2-High</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>HER2-Low</td>
<td>6/6</td>
</tr>
</tbody>
</table>

B. Serial transplantability of untreated or drug-treated xenotransplants

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Outgrowths/injections</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1^st passage (10^6 injected cells)</td>
<td>2^nd passage (10^5 injected cells)</td>
</tr>
<tr>
<td>MDA-MB-361</td>
<td>NT</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>Trastuzumab</td>
<td>3/5</td>
</tr>
<tr>
<td>SKOV-3</td>
<td>NT</td>
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<td>Trastuzumab</td>
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<tr>
<td>MCF7</td>
<td>NT</td>
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<td>Doxorubicin</td>
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<td></td>
<td>Trastuzumab</td>
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Abbreviation: NT, untreated.
High HER2 surface levels define tumor-initiating cells in HER2-overexpressing cell lines. To determine whether HER2 surface expression would define tumor-initiating cells, we sorted cells derived from MDA-MB-361 or SKOV-3, according to membrane HER2 expression levels (defined here as HER2-Low and HER2-High; Supplementary Fig. S3), and we tested SFE in the different populations.

MDA-MB-361 HER2-High cells, which represented 10% of the total cell population, showed 3-fold higher SFE than their HER2-Low counterpart (10% of total cells; Fig. 2A). Similarly, SKOV-3 HER2-High cells (11.21% of total cells) had 3-fold higher SFE than the HER2-Low cells (10.76% of total cells; Fig. 2B). These data suggest that cells presenting the highest HER2 levels contain a larger fraction of tumor-initiating cells. In line with the enrichment in tumor-initiating cells present in the HER2-High population, Western blotting analysis of the sorted MDA-MB-361 and SKOV-3 populations showed that HER2-High cells expressed the stem cell markers Oct4 and Bmi1, whereas HER2-Low cells were negative (Fig. 2C). HER2-High cell populations of both MDA-MB-361 and SKOV-3 cell lines expressed higher levels of total and active receptor tyrosine kinases as HER2 and HER1, compared with HER2-Low cells. Only MDA-MB-361 HER2-High cells showed detectable levels of HER3 but not phosphorylated in Y1289. In addition, the activation of AKT and MAPK was significantly higher in the HER2-High compared with the HER2-Low cells, whereas the total protein levels were similar in the two populations. When the MDA-MB-361 HER2-High or the SKOV-3 HER2-High cells were grown under differentiating conditions (i.e. in matrigel), HER2 cell surface expression decreased to the levels measured in the parental cells (Fig. 2D), therefore identifying the HER2-High subset as capable of recapitulating the heterogeneity of the parental cells. Overall, these data suggest that overexpression of HER family members provides survival and proliferation signals in tumor-initiating cells.

Next, we analyzed the tumorigenicity of the HER2-High cells compared with the HER2-Low cells by injecting different cell doses in nude mice and then scoring for the development of tumors. 10^4 MDA-MB-361 and SKOV-3 HER2-High cells were able to give rise to tumors (3 of 6 mice and 4 of 6 mice with tumor, respectively), whereas no tumor growth was observed in the 6 mice injected with the HER2-Low population. However, the two populations showed similar tumor growth abilities when 10^5 or 10^6 cells were inoculated (Table 1A). Thus, HER2-High cells are enriched in tumor-initiating cells.

The Notch1 pathway and HER2 expression in tumor-initiating cells. Because breast stem cell properties are tightly regulated by Notch1 signaling (24), and the activation of Notch1 signaling has been reported to increase HER2 transcription (25, 26), we analyzed Notch1 signaling in our tumor-initiating cells.

![Fig. 3. Role of Notch signaling on HER2 expression of tumor-initiating cells. A, Western blot analysis of whole cell lysates from MDA-MB-361 HER2-High/HER2-Low populations, MDA-MB-361, SKOV-3, BT-474, and ZR-75-1 spheres, and each sphere grown under differentiating conditions for Notch1 intracellular domain and Hes1 expression. MDA-MB-468 whole cell lysates were used as a positive control for Notch1 intracellular domain and Hes1 expression. B, Western blot analysis of whole cell lysates from untreated or GSI-treated MDA-MB-361 and SKOV-3 spheres for Notch1 intracellular domain and Hes1 expression. C, immunofluorescence analysis of HER2 expression in untreated (light grey line) or GSI-treated (grey line) MDA-MB-361 and SKOV-3 spheres (SPH).]
We assayed for the presence of the active form of the Notch1 receptor, the Notch1 intracellular domain, in spheres propagated in suspension or in spheres grown under differentiating conditions (Fig. 3A). Spheres derived from MDA-MB-361, SKOV-3, BT-474, and ZR-75-1 expressed higher levels of Notch1 intracellular domain compared with the spheres grown on matrigel (from 1.6 to 1.3 folds). Hes1, a known Notch1 target gene (27, 28), was up-regulated in the spheres of the four cell lines, thus confirming the activation of Notch1 in tumor-initiating cells (Fig. 3A).

Western blotting analysis revealed that MDA-MB-361 HER2-High cells, expressed 3-fold more Notch1 intracellular domain and 2-fold more Hes1 protein compared to HER2-Low cells (Fig. 3A).

To analyze the role of Notch1 pathway in regulating HER2 expression in tumor-initiating cells, we treated our cell lines with GSI, in order to block Notch1 intracellular domain formation and translocation into the nucleus (29). GSI treatment, which blocked both Notch1 intracellular domain formation and Hes1 expression in spheres (Fig. 3B), reduced the HER2 cell surface expression on MDA-MB-361 and SKOV-3 spheres by 70% and 50%, respectively (Fig. 3C), with no effect on HER2 expression in spheres grown on differentiating conditions (Supplementary Fig. S4). Accordingly, GSI treatment reduced the HER2 cell surface expression on the MDA-MB-361 HER2-High population by 7-fold whereas the reduction of HER2 expression on the HER2-Low population was minimal; mean fluorescence intensity of 483.36±324.41 versus 69.03±75.28 and 139.66±64.04 versus 106.08±58.56, respectively.

Inhibition of Notch1 intracellular domain formation led to a significant decrease in HER2 mRNA levels in the spheres formed by our four cell lines (Supplementary Fig. S5). Western blotting analysis showed that GSI treatment down-regulated HER2 expression in all carcinoma cell lines (Fig. 4A, boxed area).
Most remarkable was the 90% reduction in SFE of GSI-treated MDA-MB-361 cells ($P < 0.0001$; Fig. 4A). Similar results were obtained in GSI-treated SKOV-3 cells (60% reduction in SFE, $P = 0.0095$), in GSI-treated BT-474 (34% reduction in SFE, $P = 0.0305$), and ZR-75-1 (30% reduction in SFE, $P = 0.0434$). By contrast, GSI treatment did not affect either SFE or HER2 transcript in MCF7 cells. Strikingly, SFE resulted 5 times higher on the HER2-High compared to the HER2-Low MDA-MB-361 population and GSI treatment affected the SFE of both populations (Fig. 4B).

To validate these observations, we silenced Notch1 in MDA-MB-361, ZR-75-1, and MCF7 cells and analyzed SFE. Notch1 small interfering RNA treatment, found to reduce significantly the expression of Notch1 transcripts and HER2 mRNA level, decreased by 50% to 60% the SFE of MDA-MB-361 and ZR-75-1 cells (Fig. 4C). Our data suggest that Notch1 signaling regulates HER2 transcription in HER2-overexpressing breast carcinoma tumor-initiating cells, therefore affecting their self-renewal properties. In HER2-negative cells, Notch1 silencing did not affect HER2 mRNA expression, but was still effective in SFE reduction (Fig. 4D), suggesting that Notch1 but not HER2 is implicated in the self-renewal of these tumor-initiating cells.

Drug sensitivity of tumor-initiating cells present in HER2-overexpressing carcinoma cell lines. Because tumor-initiating cells derived from HER2-overexpressing cell lines expressed...
Fig. 6. Effect of trastuzumab and lapatinib on tumor-initiating cells of HER2-overexpressing cell lines. A, SFE of untreated (light grey bar), doxorubicin- (black bar), or trastuzumab-treated (grey bar) xenotransplants of MDA-MB-361, SKOV-3, ZR-75-1, and MCF7 cells. SFE was calculated from 6 mice/group as mean ± SD. B, Western blot analysis of spheres from untreated (NT), doxorubicin (DXR)-, or trastuzumab (TRA)-treated xenotransplants of MDA-MB-361, SKOV-3, BT-474, ZR-75-1, and MCF7 cells for expression of HER2 and the stem cell markers Bmi1 and Oct4. Markers expression was compared with spheres recovered from xenotransplants and then grown under differentiating conditions (DIFF). C, SFE analysis of MDA-MB-361, SKOV-3, BT-474, ZR-75-1, and MCF7 cells untreated (light grey bar) or treated with trastuzumab at 25 (grey bar), 50 (dark grey bar), or 100 µg/mL (black bar) cells. D, SFE analysis of MDA-MB-361, SKOV-3, BT-474, ZR-75-1, and MCF7 cells untreated (light grey bar) or treated with lapatinib at 0.01 (grey bar) or 0.1 µmol/L (black bar).
high levels of HER2 protein, we asked whether trastuzumab, the humanized monoclonal antibody directed against HER2, would be more effective than conventional chemotherapeutics, like doxorubicin, in targeting tumor-initiating cells. To this end, we s.c. injected cells derived from HER2-overexpressing cell lines into the mammary fat pad or in the flank of nude mice and monitored tumor development in mice treated with trastuzumab or doxorubicin at doses that would partially inhibit tumor growth. We found that both trastuzumab and doxorubicin inhibited MDA-MB-361, SKOV-3, BT-474 and ZR-75-1 carcinoma cell lines (data not shown). The SFE of HER2-overexpressing cell lines was significantly lower than that of doxorubicin-treated or untreated tumors. By contrast, the SFE of MCF7 xenografts were not affected by doxorubicin or trastuzumab treatment (Fig. 6A). To exclude that the differences in SFE efficiencies would simply reflect a generalized reduction in cellular viability, cells derived from xenografts of all the experimental groups were also cultured in adherent condition and cell growth was monitored by MTT assay. We found that all treated xenograft-derived tumor cells showed comparable growth when cultured in adhesion (Supplementary Fig. S6A).

Interestingly we found that levels of the stem cell markers Oct4 and Bmi1 were higher in xenotransplant-derived spheres than in the same spheres grown under differentiating conditions, thus suggesting that spheres derived from xenografts had tumor-initiating cell properties (Fig. 6B).

To functionally assess the tumor-initiating cell properties of xenograft-derived cells, residual cells were serially transplanted at different cell doses. Serial transplant analysis of MDA-MB-361 and SKOV-3 xenografts showed that cells from control or doxorubicin-treated tumors were transplantable multiple times, whereas cells derived from trastuzumab-treated tumors could not be transplanted into a secondary recipient at any of the cell doses tested (Table 1B). MCF7 cells derived from untreated, trastuzumab-, or doxorubicin-treated tumors showed similar transplantation abilities (Table 1B). In addition, we studied the effect of trastuzumab on SFE and on the growth ability of the differentiated counterparts. Treatment of MDA-MB-361, SKOV-3, and BT-474 cells caused a dose-dependent inhibition of SFE (Fig. 6C), whereas growth in differentiating conditions was only marginally affected (Supplementary Fig. S6B).

We also found that lapatinib, an inhibitor of HER2 and HER1 tyrosine kinase activity, at doses able to decrease phospho-HER2 protein levels almost by 80% in MDA-MB-361, SKOV-3, BT-474 and ZR-75-1 carcinoma cell lines (data not shown), decreased by 30% to 50% the ability of these cells to form spheres depending on the drug concentration (Fig. 6D). Of note, lapatinib treatments had mild effects on the growth ability of the differentiated counterparts (Supplementary Fig. S6C). In MCF7 cells 0.01 μmol/L lapatinib affected both SFE and growth ability in differentiating conditions.

Therefore, our data strongly suggest that anti-HER2 therapeutics, especially trastuzumab, can effectively target tumor-initiating cells derived from HER2-overexpressing tumor cell lines.

### Discussion

The present study provides evidence that in HER2-positive carcinoma cell lines, cells presenting the highest levels of the HER2 oncoprotein showed higher frequencies of tumor-initiating cells. In fact, not only cells grown as spheres or positive for ALDH activity presented higher levels of HER2, but subpopulations of cells with elevated surface expression of HER2 (HER2-High cells) also showed increased SFE compared with the bulk population of cells. HER2-High cells also had the highest ability to grow in vivo in a xenotransplantation animal model that remains the gold standard assay for testing functional stem cell properties.

The mechanism controlling HER2 expression levels in the tumor-initiating cells does not depend on variation of HER2 gene copies, but rests, at least in part, in Notch1-dependent regulation, a molecule previously shown to increase HER2 transcription and to regulate breast stem cell survival (30), as shown by the reduction of HER2 cell surface expression together with SFE following inhibition or silencing of Notch1 signaling. The lack of effect of Notch1 inhibitor on HER2 expression in HER2-overexpressing cells grown in differentiating conditions could be explained by the significantly lower levels of activated Notch1 in these cells. Also, the higher HER2 expression levels found in cells presenting ALDH activity support a role of this oncoprotein in the maintenance of the putative breast tumor-initiating cells. In analogy with several genes, including Notch, Hedgehog, Wnt, and ras, initially identified as oncogenes but shown subsequently to be involved in tumor-initiating cell pathways of self-renewal and survival (3, 31, 32), HER2 might also represent a relevant molecule in cancer stem cells. Similarly, Korkaya et al. (33) found that increased HER2 expression in normal mammary epithelial cells or in breast carcinoma cell lines enhances the tumor-initiating cell component driving tumorigenesis, invasion, and metastasis. Although no HER2 increase was observed in MCF7 spheres compared with cells grown under differentiation conditions by fluorescence-activated cell sorting, it is possible that a Notch1-dependent increase in HER2 on tumor-initiating cells was below the limits of detection in this low-HER2-expressing cell line.

Moreover the therapeutic activity of trastuzumab seems to extend its action on putative cancer stem cells of HER2-positive carcinoma cell lines and the effect is due mainly to the elevated HER2 expression in tumor-initiating cells compared with the other bulk tumor cells. Indeed, the lack of serial transplantability of residual cells derived from trastuzumab-treated HER2-overexpressing xenografts indicates that tumor cells growing in trastuzumab-exposed mice were depleted of tumorigenic cells. Higher sensitivity to trastuzumab or lapatinib of tumor-initiating cells compared with differentiated counterparts evidenced in vitro, confirms in vivo findings and suggests that stemness markers (i.e. Bmi1 and Oct4) might be predictive of tumor cell sensitivity to trastuzumab. The activity of the trastuzumab antibody in breast carcinoma patients is directly proportional to the level of HER2 present on tumor cell membrane (34). Thus, our observation of higher HER2 expression in tumor-initiating cells of HER2-overexpressing carcinoma cell lines compared with the same cells grown on matrigel explains why trastuzumab is so efficient in inhibiting the tumorigenicity of HER2-positive carcinoma cells. HER2-High cells contained a larger fraction of tumor-initiating cells,
the only population able to give rise to tumor growth at very low dilutions. In light of these results, it is even clearer why the four clinical trials involving more than 10,000 cases of HER2-positive breast carcinoma patients (9) revealed that trastuzumab represents a success in the field of targeted antineoplastic agents. More importantly, the tumor-initiating cells of breast carcinoma cell lines present higher levels of active receptors of HER family with consequent increased survival and proliferation signaling mediated by these receptors. Signaling mediated by HER family receptors drives the stem cell pool, i.e. regulating the stem/progenitor cell populations, as also supported by significant SFE reduction of breast carcinoma cell lines by inhibitor of HER1 and HER2 kinase activity at doses ineffective on differentiated cells.

In the clinical context, it is important to note that HER2 levels in tumor-initiating cells of breast carcinoma overexpressing HER2 but without gene amplification were also higher compared with total tumor cells, with levels reaching those of cells showing gene amplification. This suggests the usefulness of trastuzumab in treating these types of tumors by virtue of its ability to bind and block the cells responsible for tumor aggressiveness. In fact, benefits for disease-free survival after trastuzumab adjuvant therapy have been observed in patients with breast tumors negative for HER2 overexpression (based on fluorescence in situ hybridization criteria) or with less than 3+ staining intensity (based on HercepTest immunohistochemical scoring; ref. 35).

Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35). Our data do not exclude a potential sensitivity of tumor-initiating cells to chemotherapy; indeed, the similar SFE and scoring; ref. 35).

Although the relevance of our results obtained with tumor-initiating cell–enriched cells from carcinoma cell lines awaits confirmation in studies using tumor cells obtained from breast tumor patients, we provide evidence of the efficacy of trastuzumab in inhibiting tumor-initiating cells of HER2-positive tumors. Moreover, our finding that HER2 expression is up-modulated in tumor-initiating cells in a Notch1-dependent manner in addition to gene amplification suggests the potential for new therapeutic modalities targeting this stem cell marker pathway.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

31. Liu BY, McDermott SP, Khwaja SS, Alexander CM. The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci USA 2004;101:4158–63.
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Alessandra Magnifico, Luisa Albano, Stefano Campaner, et al.