Evolution of HER2-positive mammary carcinoma: HER2 loss reveals claudin-low traits in cancer progression

Veronica Giusti¹, Francesca Ruzzi², Lorena Landuzzi¹, Marianna L. Ianzano², Roberta Laranga², Elena Nironi², Laura Scalambra², Giordano Nicoletti¹, Carla De Giovanni², Martina Olivero³, Maddalena Arigoni⁴, Raffaele Calogero⁴, Patrizia Nanni², Arianna Palladini² and Pier-Luigi Lollini².
**Supplementary Table S1.** Number of huHER2 gene copies quantified by Real-Time PCR. $\Delta Ct = C_{huHER2} - C_{hu/mPTGER2}$. Expression level of $2^{\Delta Ct \text{ MDA-MB-231/Av} \times \Delta Ct \text{ MCF7/2}}$ was associated to two HER2 copies (n=2, at least). TS/A is a murine mammary cancer cell line.

| Cell line                     | $\Delta Ct$ | huHER2 copy number |
|-------------------------------|-------------|--------------------|
| Mambo89HER2<sup>stable</sup> | -7.27       | 51                 |
| Mambo43HER2<sup>labile</sup> | -5.83       | 19                 |
| Mambo38HER2<sup>loss</sup>   | -5.79       | 18                 |
| TS/A                          | 7.26        | 0                  |
| Non-transgenic normal tissue  | 4.25        | 0                  |
| HER2-transgenic normal tissue | -5.96       | 26                 |
| HER2-transgenic mammary tumor | -6.60       | 32                 |
| HCC1954                       | -9.34       | 215                |
| BT474                         | -7.53       | 61                 |
| SKBr3                         | -5.25       | 13                 |
| MDA-MB-453                    | -4.14       | 6                  |
Supplementary Table S2. List of kinases with significantly co-expressed genes among those up-regulated in HER2-negative cells as analysed by ArchS4 Kinase.

| Term   | Overlap | Adjusted P-value |
|--------|---------|------------------|
| DDR2   | 51      | 8.76E-30         |
| PDGFRB | 50      | 4.94E-29         |
| PDGFA  | 48      | 3.88E-27         |
| ACVR1  | 47      | 2.42E-26         |
| MYLK   | 47      | 2.42E-26         |
| RPS6KA2| 46      | 2.04E-25         |
| NEK7   | 40      | 1.03E-19         |
| LATS2  | 38      | 5.90E-18         |
| MERTK  | 33      | 9.25E-14         |
| SRC    | 33      | 9.25E-14         |
| GRK5   | 30      | 1.89E-11         |
| NEK6   | 30      | 1.89E-11         |
| ABL2   | 29      | 1.01E-10         |
| IRAK2  | 28      | 4.90E-10         |
| CAMK1  | 28      | 4.90E-10         |
| MAP3K6 | 27      | 2.46E-09         |
| DYRK3  | 26      | 1.19E-08         |
| PTK7   | 24      | 2.22E-07         |
| CSF1R  | 25      | 5.52E-08         |
| SGK1   | 24      | 2.22E-07         |
| TGFBR2 | 24      | 2.22E-07         |
| ACVRL1 | 23      | 8.39E-07         |
| FGFR1  | 23      | 8.39E-07         |
| TRIO   | 23      | 8.39E-07         |
| ABL1   | 23      | 8.39E-07         |
| ALPK2  | 22      | 3.21E-06         |
| ILK    | 22      | 3.21E-06         |
| LIMK1  | 22      | 3.21E-06         |
| TEK    | 21      | 1.22E-05         |
| SIK2   | 21      | 1.22E-05         |
| DAPK1  | 20      | 4.57E-05         |
| CDK15  | 19      | 1.49E-04         |
| DAPK3  | 19      | 1.49E-04         |
| MET    | 19      | 1.49E-04         |
| PKDCC  | 19      | 1.49E-04         |
| AXL    | 19      | 4.64E-04         |
| RIPK1  | 18      | 4.64E-04         |
| MAPK13 | 18      | 4.64E-04         |
| EGFR   | 18      | 4.64E-04         |
| MAP3K8 | 18      | 1.38E-03         |
| PLK3   | 17      | 1.38E-03         |
| RYK    | 17      | 1.38E-03         |
| CDK4   | 17      | 1.38E-03         |
| TIE1   | 17      | 3.74E-03         |
| IKBKE  | 16      | 3.74E-03         |
| Term      | Overlap | Adjusted P-value |
|-----------|---------|------------------|
| PIM3      | 16      | 3.74E-03         |
| CDC42BPB  | 16      | 3.74E-03         |
| EPHA2     | 16      | 3.74E-03         |
| MAP2K3    | 16      | 3.74E-03         |
| RIPK2     | 16      | 1.02E-02         |
| MAP4K4    | 15      | 1.02E-02         |
| PLK2      | 15      | 1.02E-02         |
| RNASEL    | 15      | 2.63E-02         |
| VRK2      | 14      | 2.64E-02         |
| ROR2      | 14      | 2.62E-02         |
**Supplementary Fig. S1.** Tumor growth of s.c. injected MamBo43HER2<sup>labile</sup> cells in different *in vivo* models. (A) MamBo43HER2<sup>labile</sup> cells were s.c. injected into FVBhuHER2 mice at different doses: 10<sup>6</sup> (circle), 10<sup>7</sup> (square), 2x10<sup>7</sup> (triangle). (B) MamBo43HER2<sup>labile</sup> cells were s.c. injected (10<sup>5</sup> cells) into immunodeficient mice (open circle) or FVBhuHER2 immunocompetent mice (solid circle). Data showed mean and SEM of 2-3 mice. (C) HER2 expression in tumors growing in immunocompetent mice as in picture (A) and in immunodeficient mice as in picture (B) detected by cytofluorometric analysis. Each bar represents the mean and SEM of 2-3 tumors.
Supplementary Fig. S2. HER2 expression by MamBo cell lines. (A) Level of HER2 transcript by Real-Time PCR; MamBo89HER2\textsuperscript{stable} and MamBo43HER2\textsuperscript{labile}, n=2; MamBo38HER2\textsuperscript{loss}, n=4. Each bar shows mean and SEM. \(\Delta Ct = Ct_{\text{hu}HER2} - Ct_{\text{mTBP}}\). (B) Levels of HER2 protein and of its phosphorylated isoform pHER2 detected by Western blot.
Supplementary Fig. S3. Effect of demethylation on MamBo38HER2<sup>lo</sup> cell line. Cells were seeded at 8x10^4 cells/cm<sup>2</sup> and treated with 5-aza-2'-deoxycytidine 0.5 μM (square) or 5 μM (triangle) or medium only (circle). (A) Cell growth. (B) HER2 expression evaluated by flow-cytometry and reported as fluorescence intensity (MFI).
Supplementary Fig. S4. Trastuzumab treatment in MamBo43HER2labile cells in vitro and in vivo. (A) Sensitivity on 2D culture after 120h of treatment. Each bar showed the mean and SEM; n=4. (B) HER2 expression of untreated (black profile) and trastuzumab-treated (10 µg/ml) (red profile) cells measured by cytofluorometric analysis. (C) Trastuzumab effect in vivo (4 mg/Kg) on the growth of tumors induced by subcutaneous injection of MamBo43HER2labile cells. Vehicle, black circles; trastuzumab 4 mg/Kg, red squares; n=4-5 mice. *p<0.05 vs vehicle, by Student’s t-test.
Supplementary Fig. S5. EMT profile by Real-Time PCR of MamBo43HER2labile, trastuzumab-treated MamBo43HER2labile (MamBo43HER2labile TRT) and MamBo38HER2loss cell lines respectively n=3, n=4 and n=2, at least. ΔCt=ChuHER2-CmTBP. Each bar shows mean and SEM. *p<0.05; **p<0.01; ***p<0.001, by unpaired t test with Welch’s correction.
Supplementary Fig. S6. Sunitinib activity in vivo on MamBo38HER2<sup>low</sup> cells. (A) Growth under 2D-adherent conditions. n=3. (B) Growth under 3D non-adherent conditions of sunitinib-treated cells (1 and 5 μM). * p<0.05 versus vehicle. Respectively, by Student’s t-test. n=4 (C) Sunitinib (5 μM) modified morphology of MamBo38HER2<sup>low</sup> cells. Bar is equivalent to 200 μm. (D) E-cadherin expression by Real-Time PCR of untreated (green), vehicle (green with rows) and sunitinib-treated (5 μM) cells (solid orange). Data reported mean and SEM. ΔCt=C<sub>huHER2</sub>-C<sub>mTBP</sub>. n=2. *p<0.05 by Student’s t-test.