Expression profile of tyrosine phosphatases in HER2 breast cancer cells and tumors

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Abstract. Background: HER2-overexpression promotes malignancy by modulating signalling molecules, which include PTPs/DSPs (protein tyrosine and dual-specificity phosphatases). Our aim was to identify PTPs/DSPs displaying HER2-associated expression alterations.

Methods: HER2 activity was modulated in MDA-MB-453 cells and PTPs/DSPs expression was analysed with a DNA oligoarray, by RT-PCR and immunoblotting. Two public breast tumor datasets were analysed to identify PTPs/DSPs differentially expressed in HER2-positive tumors.

Results: In cells (1) HER2-inhibition up-regulated 4 PTPs (PTPRA, PTPRK, PTPN11, PTPN18) and 11 DSPs (7 MKPs [MAP Kinase Phosphatases], 2 PTP4, 2 MTMRs [Myotubularin related phosphatases]) and down-regulated 7 DSPs (2 MKPs, 2 MTMRs, CDKN3, PTEN, CDC25C); (2) HER2-activation with EGF affected 10 DSPs (5 MKPs, 2 MTMRs, PTP4A1, CDKN3, CDC25B) and PTPN13; 8 DSPs were found in both groups. Furthermore, 7 PTPs/DSPs displayed also altered protein level. Analysis of 2 breast cancer datasets identified 6 differentially expressed DSPs: DUSP6, strongly up-regulated in both datasets; DUSP10 and CDC25B, up-regulated; PTP4A2, CDC14A and MTMR11 down-regulated in one dataset.

Conclusions: Several DSPs, mainly MKPs and, unexpectedly, MTMRs, were altered following HER2-modulation in cells and 3 DSPs (DUSP6, CDC25B and MTMR11) were altered in both cells and tumors. Among these, DUSP6, strongly up-regulated in HER2-positive tumors, would deserve further investigation as tumor marker or potential therapy target.

Keywords: Tyrosine phosphatases, phosphorylation, HER2, breast cancer, array analysis

1. Introduction

About 10% of human breast cancers display overexpressed HER2 (human epidermal growth factor receptor, ERBB2), a member of the ERBB receptor family [13,17]. Such tumors are highly invasive and metastatic, and generally do not respond to anti-estrogen treatments [28,29]. Studies in tumors and tumor-derived cell lines indicated that HER2 overexpression is mainly due to gene amplification. HER2 repression, using tyrosine kinase inhibitors or monoclonal antibodies, is a valuable therapeutic approach [3,20]. HER2 activation evokes stimulation of both MAPK and PI3K/AKT pathways, leading to proliferation and survival [40]. Also pathways governing cell motility and invasion may be up-regulated [30,39,42]. However, the way HER2 over-expression deregulates these multiple signaling pathways is still poorly understood [19,39]. Within this background, PTPs (protein tyrosine phosphatases) and DSPs (dual-specificity phosphatases) might play a role, since members of these families are signaling molecules in RTK (receptor tyrosine kinase) pathways.

The human genome sequence revealed that only 38 PTPs exist, namely 21 receptor and 17 soluble PTPs. DSPs are approximately 65 [32] and include members of the MKPs, SSH, PTP4A, CDC14, PTEN, Myotubularins, CDC25 and EYA families [1,2]. PTPs and DSPs are positive or negative regulators in a wide range of signaling networks, thus contributing to control cell proliferation, adhesion and migration, differentiation and survival [15,32].

In cancer pathways, PTPs and DSPs generally play an anti-oncogenic role, by terminating the action of
PTKs (protein tyrosine kinases). Examples are the MKPs (i.e., DSPs that dephosphorylate MAPKs) [14] and several PTPs [32]. However, PTPs may also transduce positive signals, thus favoring oncogenesis. Examples are PTPN11 [23], PTPN1 [33], PTPRE [11] and the cell cycle regulators CDC25C, B and A [6]. In any event, PTPs and DSPs must be regulated to prevent havoc in the cell. Little is still known on PTPs and DSPs in breast cancer, particularly in the HER2-dependent type. Several studies focused on PTEN, a tumor suppressor which is often mutated in tumors [25]. A role in favoring HER2-evoked tumorigenesis was also reported for PTPN11 [21,43] and PTPN1 [4]. Other studies indicated a role for PTPN18 [10] in HER2-overexpressing tumor cell lines and for PTPRE in neu mice [11]. However, many more PTPs and DSPs might be involved, given the complexity of the ERBB receptors signaling.

PTPs and DSPs alterations in cancer may comprise changes in the expression and protein levels, or mutations, as described in the case of colon cancer [38]. Post-translation modifications may also occur. The present project was undertaken to identify PTPs and DSPs displaying altered expression levels in HER2-overexpressing human breast cancer and cancer-derived cell lines. We analyzed the PTPs and DSPs in: (1) MDA-MB-453 cells (HER2-overexpressing human breast cancer cells) carrying permanent HER2 inhibition or activated with EGF and (2) 2 public datasets of primary invasive HER2-positive breast tumors. The aim was to identify PTPs and DSPs displaying HER2-associated alterations, that might sustain tumor biology features, such as survival and motility, and might represent new tumor markers or potential therapy targets.

2. Materials and methods

2.1. Materials

Tissue culture media and additives, fetal calf serum, EGF, the antibodies to α-tubulin, peroxidase-conjugated antibodies and protein A-peroxidase were purchased from Sigma Aldrich (Munich, Germany). The other commercial antibodies were as follows: phospho-Y1248-HER2, pan-EGFR, DUSP1, DUSP2, DUSP6, MTM1 and PTEN (Santa Cruz Biotech, Heidelberg, Germany); pan-HER2 (Cell Signaling Technology, Danvers, MA, USA); phosphoY1173-EGFR (Nanotools, Teningen, Germany); PTPN11 (Upstate, Lake Placid, NY, USA) and CDKN3 (BD Biosciences, Franklin, NJ, USA). The anti-CDC25C antibodies were described previously [35]. The anti-PTPRA rabbit polyclonal antibodies were a kind gift from Dr. J. den Hertog (Utrecht, The Netherlands).

2.2. Cell culture, treatments and extracts

The MDA-MB-453 cells were grown in RPMI supplemented with 10% fetal calf serum [36]. The MDA-MB-453 cells carrying the intracellular expression of the p185HER2-specific single chain antibody (5R) and control MDA-MB-453 cells carrying the empty vector (LSBN) were grown in DMEM supplemented with 10% fetal calf serum and 0.5 mg/ml geneticin [8]. In brief, cDNA encoding the p185HER2-specific single chain antibody 5R was cloned into the LXSN retroviral vector and used to infect the packaging cell line gp + AM12. Following G418 selection the virus-containing medium was collected and used to infect the MDA-MB-453 cell line. The infection cycle was repeated three times and cells were tested for p185HER2 surface expression by FACScan analysis. For EGF-treatment, sub-confluent cells were serum-starved and exposed to 50 ng/ml EGF (diluted in DMSO) or to DMSO alone (time 0). For immunoblotting, the cells were washed in cold PBS and lysed in 50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 7.5 mM 2-mercaptoethanol, added with 1 mM orthovanadate, 50 mM NaF and protease inhibitors (0.02% benzamidine, 0.02% PMSF, 0.02% TPCK, 10 µg/ml soybean trypsin inhibitor and 4 µg/ml leupeptine). Cell extracts were then diluted 1:2 in Laemmli buffer and boiled.

2.3. RNA extraction and cDNA/cRNA preparation

RNA was purified from cells grown on 30 mm plates using the SV total RNA isolation system (Promega, Madison, WI, USA). Labeled antisense RNA (cRNA) was synthesized using the TrueLabeling-AMP 2.0 kit (SABiosciences, formerly SuperArray Biotech, Frederick, MA, USA). In brief, cDNA was prepared by reverse transcription of total RNA (2–3 µg) and immediately used for cRNA synthesis, labeling and amplification in the same tube. All the rest was according to the kit instructions. Four h incubation yielded between 4 and 12 µg of cRNA.
2.4. DNA arrays

Custom oligo GEArray, displaying 60-mer oligonucleotide probes, targeting 103 PTPs and DSPs and 5 housekeeping genes (RPS27A, GAPDH, B2M, HSP90AB1 and ACTB), were produced by SABiosciences. cRNA (2 µg) was used for overnight hybridization, followed by peroxidase-based chemiluminescence detection on X-ray films, according to the manufacturer’s instructions. Data extraction, normalization with the indicated housekeeping genes and analysis used the GEArray Expression Analysis Suite (SABiosciences). Expression levels are given as the ratio of treated to control cells and we reported changes greater than 30%, a boundary selected to boost the results reliability.

2.5. Quantitative PCR

cDNA was prepared by reverse transcription of total RNA (2 µg) using the ReactionReady First Strand cDNA Synthesis kit (SABiosciences). Real time PCR was performed on Applied Biosystems instruments (Foster City, CA, USA), according to the manufacturer’s instructions, using the ABI Prism 7900HT Sequence detection system (Applied Biosystems). Primers were designed using Primer Express software (Applied Biosystems). Reactions contained 10 µl of SYBR Green PCR Master mix, 5 pmol of each primer, 5 ng of cDNAs in a final volume of 20 µl. PCR runs were as follows: initial denaturation at 95°C for 10 min, 40 amplification cycles including denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min. At the end of the 40 PCR cycles, the temperature, Tm, at which the dsDNA amplicon and intercalated SYBR green complex is denatured, was determined by continuously recording the fluorescence during progressive heating up to 95°C, with a ramp rate of 0.1°C/s. Normalization was performed with GAPDH and ACTB. Gene expression levels were calculated using the comparative Ct method.

2.6. Immunoblotting

Electrophoresis on 8.5% polyacrylamide-SDS gel was followed by transblotting onto Immobilon-P (Millipore, Billerica, MA, USA) [37] and probing with the indicated antibody. The detection system used protein A-peroxidase or peroxidase-conjugated secondary antibody and the Lumilin chemiluminescence kit (Santa Cruz Biotech). For antibody re-probing, the membranes were previously treated with 5 mM phosphate buffer, 2% SDS and 2 mM 2-mercaptoethanol at 60°C for 15 min. For immunoblot quantification, the PTP or DSP protein bands from treated and control cells were analyzed by densitometric scanning and normalized to the α-tubulin band analyzed in the same experiment (i.e., the PTP or DSP/α-tubulin value ratios were calculated). Normalized data from different experiments were averaged, as specifically indicated. Results are given as percent of averages from control cells (controls were LBSN cells in Fig. 1 and time 0 cells in Fig. 3).

2.7. Public gene expression data analysis

The expression data from the van de Vijver et al. dataset [34] were downloaded from http://www.rii.com/publications/2002/nejm.html. Such data were generated by hybridization on arrays containing 25,760 oligonucleotides, corresponding to 24,479 genes, of Cy-labeled cRNA from a tumor sample, and the reverse-color Cy-labeled product from a pool that consisted of an equal amount of cRNA from each patient. The expression and clinical data from Naume et al. [22] were recovered from GEO (GSE3985). The data were generated by hybridization of Cy5-labeled cRNA from tumor and Cy3-labeled cRNA from the Universal Human Reference (Stratagene, La Jolla, CA, USA) on cDNA arrays containing 42,000 features, representing 24,271 unique cluster IDs (UniGene Build-Number173) produced at the Stanford Functional Genomics Facility (http://www.microarray.org/sfgf/jsp/home.jsp). Hierarchical clustering was performed and displayed using Cluster and TreeView softwares (http://www-stat.stanford.edu/~tibs/). Class comparisons were performed using BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html).

3. Results

3.1. PTPs and DSPs expression in MDA-MB-453 cells following HER2-modulation

To analyze PTPs and DSPs in MDA-MB-453 cells (a human HER2-overexpressing breast tumor cell line) we designed a DNA oligo-array targeting all the 38 PTPs and 65 DSPs of the human genome.
3.1.1. HER2 inhibition

HER2 was inhibited due to the stable intracellular expression of an anti-HER2 antibody (5R cells, described by [31] and control cells carrying the empty vector, LBSN cells). 5R cells displayed impaired HER2 membrane localization [8] and HER2 inactivation (Fig. 1C). Twenty two PTPs and DSPs genes were found altered in 5R cells, namely 15 up-regulated and 7 down-regulated, with respect to control cells (Table 1). The up-regulated genes included 4 PTPs (the receptor PTPRA and PTPRK, the cytosolic PTPN11 and PTPN18) and 11 DSPs (7 MPK, 2 PTP4A and 2 MTMR genes). The down-regulated genes were all DSPs (including 2 MKP and 2 MTMR genes). Altogether, the vast majority of altered genes were DSPs genes, MKP being the most represented family. A randomly chosen sampling of the altered genes (9 out of 22) was further investigated by Real-Time PCR. The alterations detected by the array analysis were overall confirmed (Table 1).

Since RNA alterations are not always followed by protein changes, we investigated also the protein level of 9 of the altered PTPs and DSPs. Immunoblot analysis indicated that all the down-regulated genes displayed also significant protein decrease, whereas among the up-regulated genes, only DUSP1 displayed increased protein (Fig. 1A and B).

Overall, the results indicated that stable HER2 inhibition affected the expression of about 1/5 of the PTP and DSP genes analyzed (22 out of 103). The majority of the altered genes (18 out of 22) were DSPs and the up-regulated genes (15) prevailed over the down-regulated ones (7). A matching change in the protein level occurred in the case of all the down-regulated genes tested, but seemed much less frequent among the up-regulated ones.

3.1.2. HER2 stimulation

Due to the lack of HER2 ligands, to stimulate HER2 we exploited its ability to form HER2-EGFR (EGF receptor) heterodimers, which can be stimulated with EGF [7,12]. Treatment of MDA-MB-453 cells for up to 18 h indicated that HER2 activation peaked at 5 min and persisted throughout the stimulation, whereas EGFR activation was maximal at 10 min and decreased subsequently (Fig. 2C). For DNA microarray expression analysis, total RNA was obtained from non-stimulated cells and from cells stimulated for 2, 12 or 18 h. To subtract the possible contribution from the EGFR homodimers, we treated also 5R cells (in these cells, due to HER2 inhibition, the phosphatase alterations induced by EGF would originate only from the EGFR homodimers). The genes altered in 5R cells were then removed from the list of the genes altered in the EGF-stimulated MDA-MB-453 cells. The remaining gene alterations were due to EGF stimulation of the HER2-EGFR heterodimers. Eleven genes (10 DSPs and PTPN13) were found altered with respect to time 0 and were further divided into four groups, based on the temporal pattern displayed (Fig. 2A). The DSPs belonging to the MKP family (namely DUSP1, DUSP6 and STYXL1, panel a; DUSP12 and DUSP16, panel b) were all up-regulated at 12 h and some also at 18 h (panel a). The remaining genes displayed up-regulation at 2 h, followed (CDKN3 and PTP4A1, panel c) or not (PTPN13, MTM1 and MTMR6, panel d) by decrease. CDC25B was constantly decreasing (panel c). Six genes, randomly chosen out of the 11 altered genes, were further investigated by Real-Time PCR at time 0, 2 and 12 h of EGF stimulation. The results generally confirmed the alterations detected by the array analysis (Fig. 2B).

The protein level of 4 out of the 11 altered PTPs and DSPs were analyzed by immunoblot. Due to the time lag presumably existing between RNA and protein response, the extracts were prepared from cells collected at 6, 12, 18 and 24 h of exposure to EGF. Time 0 cells were untreated controls. DUSP1 increased from 12 h on, and DUSP6 increased at 18 and 24 h, whereas CDKN3 decreased constantly from 6 h on (Fig. 3). Such results confirmed, though with some pattern discrepancies, the microarray data. On the other hand, MTM1 and CDC25B proteins did not change significantly. Altogether, HER2 stimulation altered the protein level of 3 out of the 5 DSPs investigated, suggesting a role for the altered proteins in the HER2-driven signaling.

3.2. PTPs and DSPs expression in HER2-positive breast tumors

About 10% of breast tumors display overexpressed HER2. To evaluate whether PTPs and DSPs were modulated in HER2 positive tumors, we analyzed 2 large public datasets of primary invasive breast tumors, both including a significant cohort of HER2-positive tumors: the van de Vijver [34] and the Naume [22] datasets, displaying expression data from 295 and 123 patients, respectively. To identify HER2-positive tumors, we selected a list of 14 genes located on chromosome 17, along the region amplified in HER2 positive tumors (not shown), and studied their expression profile in breast tumors by unsupervised hierarchical clus-
Fig. 1. PTPs and DSPs protein in MDA-MB-453 cells carrying inhibited HER2. (A) Protein immunoblots. Extracts were prepared from cells in which HER2 was inhibited by the stable expression of an anti-HER2 antibody (5R) and from control cells (carrying the empty vector, LBSN), and used to immunodetect the indicated PTP or DSP. α-tubulin was subsequently detected on the same membrane. (B) Immunoblot quantification. Protein bands as in A were quantified by densitometric scanning and normalized using α-tubulin. Results are given as percent of bands from control (LBSN) cells. Average ± SEM of 3–4 experiments performed using 3 different cell extracts. Statistical analysis (Student’s t-test): *p < 0.05; **p < 0.01. (C) HER2 inhibition in 5R cells. HER2 activity was assessed in extracts from 5R and LBSN cells by the phosphorylation level of Tyr1248 (pY1248-HER2). HER2 protein level (pan-HER2) and α-tubulin are also shown. The data are representative of 3 independent experiments.
### Table 1

PTPs and DSPs expression in HER2-inhibited MDA-MB-453 cells (5R cells)

| Symbol   | Gene name              | RNA fold change \(^{1}\) (5R/LBSN cells) |
|----------|------------------------|------------------------------------------|
|          |                        | Array | RT-PCR |
| Up-regulated genes                   |                                        |
| PTPRA   | RTP-alfa-PTPHEPTP/HLPR | 1.76  | –      |
| PTPRK   | R-PTP-kappa            | 1.36  | –      |
| PTPN11  | SHP2/Syp               | 1.31  | 1.53   |
| PTPN18  | BDPI/PTP20/PTP-HSCF    | 1.75  | –      |
| DUSP1   | CL100/MKP-1/14PTPN10   | 1.91  | –      |
| DUSP2   | PAC-1/PAC1             | 1.51  | 1.25   |
| DUSP5   | DUSP/HVH3/B23          | 1.59  | 1.51   |
| DUSP12  | DUSP12/HVYH1           | 1.79  | –      |
| DUSP16  | MKP-7/MKP7             | 1.95  | –      |
| DUSP23  | MOSP                   | 1.64  | –      |
| STYXL1  | DUSP24/MK-STYX        | 2.16  | 1.65   |
| PTP4A1  | PRL-1                  | 1.68  | 3.56   |
| PTP4A3  | PRL-3                  | 2.41  | 2.25   |
| MTM1    | MTM1/myotubulinarin    | 1.51  | 1.92   |
| MTMR12  | 3-PAP/MTMR12/PIP3AP    | 3.51  | –      |
| Down-regulated genes                  |                                        |
| DUSP6   | MKP3/PYST1             | 0.51  | –      |
| DUSP14  | MKP-L/MKP6             | 0.68  | –      |
| CDKN3   | KAP/CDI1/CIP2          | 0.19  | –      |
| PTEN    | PTEN/BZS/MHAM          | 0.49  | –      |
| MTMR4   | MTMR4                 | 0.61  | –      |
| MTMR11  | MTMR11CRA             | 0.41  | 0.65   |
| CDC25C  | CDC25C                | 0.67  | 0.73   |

**Note:** \(^{1}\) Total RNA was purified from HER2-inhibited (5R) and control (LBSN) cells and used to generate labeled antisense RNA for DNA microarray hybridization and cDNA for Real-Time PCR. The results are given as expression rates of 5R/LBSN cells. Average of 2 experiments, performed with RNA derived from two independent purifications.

In each dataset, a gene cluster representative of HER2-positive tumors was identified, principally driven by ERBB2 and GRB7 genes. Based on clustering results, 63 tumors in the van de Vijver dataset (21% of the total tumors) and 40 tumors in the Naume dataset (32% of the total tumors) were identified as HER2-positive tumors. The HER2-positive cluster in the Naume dataset included all the tumors that showed amplification of ERBB2 gene by FISH (Fluorescence in situ hybridisation) analysis. Class comparison analysis (using BRB software) identified 947 genes differentially expressed in HER2-positive versus HER2-negative tumors in the van de Vijver dataset and 351 in the Naume dataset (significant at 0.001 level of the univariate test).

Six DSP genes were found modulated in HER2-positive tumors. DUSP6 was strongly up-modulated in both datasets (\(p\)-value comparable to those of ERBB2 and GRB7 genes; Table 2 and Fig. 4). The other differentially expressed DSPs were: DUSP10 and CDC25B, up-modulated in the van de Vijver dataset; PTP4A2 and CDC14A, down-modulated in the van de Vijver dataset; and MTMR11, down-modulated in the Naume dataset (MTMR11 was not included in the van de Vijver platform; Table 2). Altogether, the analysis indicated that several DSPs were modulated in association with HER2 activation and 3 of them, DUSP6, MTMR11 and CDC25C, overlapped with the DSPs altered in MDA-MB-453 cells.

### 4. Discussion

The present work reports the first genome-wide attempt to correlate PTPs and DSPs expression and HER2 activity in a cell line. Moreover, analysis of
Fig. 2. PTPs and DSPs expression in MDA-MB-453 cells stimulated with EGF. (A) DNA microarray. Cells were serum-starved and treated with 50 ng/ml EGF. RNA purification and array procedures were as in Table 1. The genes altered due to stimulation of the HER2-EGFR heterodimers (analyzed as further detailed in the Results section) were divided into 4 groups (a–d), according to the temporal patterns displayed. The results are given as fold expression changes in stimulated cells with respect to cells at time 0. Mean values of 2 independent experiments performed with RNA derived from independent cell treatments. (B) Real-Time PCR. The expression of 6 genes was evaluated using cDNA obtained from the same RNA used for the microarray analysis. Results are fold change relative to time 0. (C) HER2 and EGFR activation. Cell extracts, prepared at the indicated time-points, were used to assess the activation of HER2 (pY1248-HER2) and EGFR (pY1173-EGFR). HER2 and EGFR are also shown. The data are representative of 3 independent experiments.
breast tumor array data revealed that 6 DSPs were modulated in HER2-positive tumors, including the DUSP6 gene, which was up-modulated in both datasets with a significance comparable to ERBB2 and GRB7 genes. A rather unexpected result was also the involvement of MTMR11, a member of a DSP family that had never been associated with tumors. In cells, stable HER2 inhibition altered the expression of 22 PTPs and DSPs, namely 18 DSPs and only 4 PTPs. Conversely, HER2 activation with EGF altered 11 PTPs and DSPs, namely 10 DSPs and only 1 PTP, some of which displayed kinetics involving both up and down-modulation. Eight DSPs were altered in both inhibited and EGF-stimulated cells and 3 out of the 6 DSPs mod-

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Fig. 3. PTPs and DSPs protein in MDA-MB-453 cells stimulated with EGF. (A) Protein immunoblots. Extracts from MDA-MB-453 cells, treated with EGF as in Fig. 2, were used to immunodetect the indicated DSPs and α-tubulin, on the same membrane. (B) Immunoblot quantification. Protein bands as in A were quantified by densitometric scanning and normalized using α-tubulin. Results are percent of bands from time 0 cells. Average of 2–3 experiments performed using different cell extracts.
PTPs and DSPs differentially expressed in HER2-positive breast tumors

| Tumor dataset | Gene  | Up-regulated genes | Down-regulated genes |
|---------------|-------|--------------------|----------------------|
|               |       | p-value¹ | FDR² | p-value | FDR  |
| van de Vijver | DUSP6 | 6.0 × 10⁻⁷ | 9.7 × 10⁻⁵ | 7.5 × 10⁻⁵ | 4.1 × 10⁻³ |
|               | DUSP10| 4.5 × 10⁻⁵ | 2.7 × 10⁻³ | 1.5 × 10⁻⁴ | 6.9 × 10⁻³ |
|               | PTP4A2|        |      | 3.4 × 10⁻⁴ | 1.8 × 10⁻² |
|               | CDC14A |        |      |          |      |
|               | CDC25B| 4.5 × 10⁻⁴ | 1.5 × 10⁻² |        |      |
|               | ERBB2 | <1 × 10⁻⁷ | <1 × 10⁻⁷ |        |      |
|               | GRB7  | <1 × 10⁻⁷ | <1 × 10⁻⁷ |        |      |
| Naume         | DUSP6 | 1.8 × 10⁻⁵ | 4 × 10⁻³  |        |      |
|               | MTMR11|        |      |          |      |
|               | ERBB2 | <1 × 10⁻⁷ | <1 × 10⁻⁷ |        |      |
|               | GRB7  | <1 × 10⁻⁷ | <1 × 10⁻⁷ |        |      |

Notes: ¹ The class comparison analysis between HER2 positive and HER2 negative tumor samples was performed using a two-sample t-test with a significance level of each univariate test of 0.001. ² FDR – False Discovery Rate. ³ ERBB2 (HER2) and GRB7, a gene belonging to the HER2 signaling and displaying co-amplification with ERBB2, are also shown, for comparison.

The involvement of these genes in HER2 signaling will deserve further investigation, adopting additional cell systems and HER2-driven tumors.

Analysis of the protein levels of 9 of the 22 genes altered in 5R cells indicated decreased protein in all the 4 down-regulated genes tested, whereas, except for DUSP1, up-regulation was not followed by protein increase. The finding that RNA up-regulation was not necessarily followed by protein increase may be indicative of the tight regulation controlling the protein level of PTPs and DSPs, as reported also in the case of PTPN11 [41].

The most frequently altered DSP family are the MKPs, involving enzymes that regulate members of all three MAPK families [24]. This may not be surprising since, among their potential substrates, ERK1/2 are growth-factor stimulated and are induced in cell proliferation, whereas p38 and JNK are involved in tumor suppression and apoptosis, all processes related to breast cancer [14]. A prominent role is played by DUSP6, which was strongly up-regulated in both tumor datasets, induced by EGF in MDA-MB-453 cells and down-regulated in 5R cells. Moreover, in cells the alteration affected also the DUSP6 protein level. Such a tight regulation might be linked to the strict substrate specificity for ERK2 and suggests the need for further investigation, aimed to clarify if DUSP6 may represent a marker in HER2-driven breast tumors. A similar expression analysis of PTPs and DSPs in SKOV3 cells (HER2-driven ovary cancer cells) carrying stable HER2 silencing, indicated several overlaps with 5R cells, which however did not include DUSP6 (EVM, unpublished observations). This data supports the hypothesis of a specific involvement of DUSP6 in breast cancer. Interestingly, also DUSP14, down-regulated in 5R cells, is among the MPKs influencing ERK2 response [9]. DUSP1 was found down-regulated also in colorectal cancer and to mediate breast cancer chemoresistance through JNK targeting, suggesting a complex regulation of different MKP cascades [14]. Also the up-regulation of DUSP10 in one tumor dataset may suggest a role for this little-known enzyme.

The second most represented DSP family are the myotubularins, displaying alteration of 5 members, including MTMR11, down-modulated in one tumor dataset and in 5R cells. This is a rather unexpected result, since up to now this family of lipid phosphatases has been associated only with myopathies and neurodegenerative diseases [27]. However, some MTMRs regulate vesicular trafficking by affecting the levels of the trafficking-mediator PtdInsP and MTMR4 expression was shown to inhibit EGFR degradation [18]. Recently, trafficking of EGFR fragments to the nucleus and to signaling pathways was proposed as a new mechanism in EGFR signaling [18]. Therefore, the contribution of MTMRs to HER2-related tumors may represent an intriguing direction for future studies.

The role of PTP4A3 in breast cancer metastasis is well-documented and the additional involvement of PTP4A1 and PTP4A2 in cell proliferation and invasion...
Fig. 4. PTPs and DSPs expression profile in primary breast tumors. Heat map of the expression profile of the ERBB2 (HER2) and GRB7 genes and the PTPs and DSPs genes modulated in breast carcinomas from van de Vijver dataset (A) and Naume dataset (B). A magnified view of the expression profiles of HER2-positive tumors is also shown.

[5] suggested their prognostic use in breast cancer [26, 32]. Our findings of PTP4A2 down-modulation in one tumor dataset and of PTP4A1 and PTP4A3 alteration in cells suggest their roles in HER2 signaling and in the cross-talk with the invasion/migration signaling.

Among the other altered DSPs, PTEN is a well-known tumor-suppressor in breast and other cancers [25] and CDKN3 was found overexpressed in breast cancer [16]. Our data on the decrease in PTEN and CDKN3 proteins following HER2-inhibition may support their link to HER2 signaling.

Surprisingly, only 4 PTPs were altered in 5R cells and 1 PTP upon HER2 stimulation. PTPRA and PTPRK are involved in cell motility [15], a function that is modulated by HER2. PTPN18 was reported as a negative regulator of HER2 signaling [10], which might account for its up-regulation in 5R cells. The only PTP induced by EGF was PTPN13, a PTP recently indicated as part of a negative feed back-loop regulating HER2 [44].

In conclusion, we identified alteration of genes that had never been linked before to HER2 signaling or
HER2-driven tumors, such as the several members of the MTMR family. Also the involvement of MKPs was surprising for the large number of genes involved and the presence of some less-known members, such as DUSP5, DUSP23 or STYXL1. Finally, the marked alteration of the ERK2 regulator DUSP6 in both tumors and cells suggests the need for further investigation, aimed to clarify the role of DUSP6 in HER2 signaling and its contribution to oncogenesis. Such studies might lead to propose DUSP6 as a new tumor marker or therapy target in tumors displaying overexpressed DUSP6.

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