The HER2 amplicon includes several genes required for the growth and survival of HER2 positive breast cancer cells

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ABSTRACT

About 20% of breast cancers are characterized by amplification and overexpression of the HER2 oncogene. Although significant progress has been achieved for treating such patients with HER2 inhibitor trastuzumab, more than half of the patients respond poorly or become resistant to the treatment. Since the HER2 amplicon at 17q12 contains multiple genes, we have systematically explored the role of the HER2 co-amplified genes in breast cancer cell growth and their relation to trastuzumab resistance. We integrated aCGH data of the HER2 amplicon from 71 HER2 positive breast tumors and 10 cell lines with systematic functional RNA interference analysis of 23 core amplicon genes with several phenotypic endpoints in a panel of trastuzumab responding and non-responding HER2 positive breast cancer cells. Silencing of HER2 caused a greater growth arrest and apoptosis in the responding compared to the non-responding cell lines, indicating that the resistant cells are inherently less dependent on the HER2 pathway. Several other genes in the amplicon also showed a more pronounced effect when silenced; indicating that expression of HER2 co-amplified genes may be needed to sustain the growth of breast cancer cells. Importantly, co-silencing of STARD3, GRB7, PSMD3 and PERLD1 together with HER2 led to an additive inhibition of cell viability as well as induced apoptosis. These studies indicate that breast cancer cells may become addicted to the amplification of several genes that reside in the HER2 amplicon. The

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1. Introduction

Breast cancer is a heterogeneous disease with several different sub-diseases with differences in tumor biology, histopathology and prognosis (Perou et al., 2000; Russnes et al., 2010; Sorlie et al., 2001). About 20% of all breast cancers are characterized by an amplification of 17q12–q21, leading to overexpression of the epidermal growth factor receptor 2, ERBB2/HER2/neu (Wolff et al., 2007). HER2 is a transmembrane tyrosine kinase with oncogenic potential and belongs to the epidermal growth factor receptor family (EGF). The family consists of four members, EGER-1 (HER1), HER2, HER3 and HER4. HER receptors form hetero- and homodimers resulting in phosphorylation and activation of their intracellular tyrosine kinase domains. This activates downstream pathways such as PI3K/AKT and RAS/MAPK regulating cell growth, survival, migration and proliferation that have a key role in cancer development (Hynes and Lane, 2005). Therapies using antibodies or small molecules targeting HER2 and its signaling pathways have emerged during the past decade. Commonly used in both primary and metastatic setting is trastuzumab (Herceptin), a monoclonal antibody against the extracellular domain of HER2. Trastuzumab has been indicated to disrupt ligand independent HER2/HER3/PI3K complexes by binding to HER2, and thus inhibits the proliferation and survival of the tumor cells by downregulating AKT signaling (Junttila et al., 2009). Other proposed mechanisms of action include induction of antibody-dependent cellular cytotoxicity, especially in patients with early-stage disease (Gennari et al., 2004). However, over half of the HER2þ patients become resistant to this treatment or show no response at all. This suggests that inhibition of HER2 alone is not sufficient for these patients and that other genes and signaling pathways may be driving cancer progression and contributing to the aggressive behavior of these tumors. HER2 independent activation of the PI3K pathway has been suggested to play a role in the lack of response to trastuzumab in the HER2þ cancers. Activating mutations in the catalytic subunit of PI3K, PIK3CA, as well as low expression levels of PI3K pathway inhibitor PTEN, have been found associated with lack of response in cell lines, and as indicators of poor prognosis in HER2þ clinical samples (Berns et al., 2007; Junttila et al., 2009; O’Brien et al., 2010; Stemke-Hale et al., 2008). Lapatinib is a dual HER1/HER2 inhibitor targeting the tyrosine kinase activity in the cytoplasmic domain, however it has been shown that tumors develop resistance mechanism also against Lapatinib (Campone et al., 2011). The combination of trastuzumab and Lapatinib treatment has shown promise in clinical trials (Baselga et al., 2012).

Since the identification of HER2 amplification in breast cancer, multiple genes have been reported co-amplified with HER2 (Kauraniemi and Kallioniemi, 2006). One of the first observations was association of TOP2A expression with overexpression of HER2 (Jarvinen et al., 1996). Kauraniemi et al. studied a 2 Mb region at the 17q12–q21 using FISH and RT-PCR and identified a common region of amplification in primary breast tumors to include six genes which were amplified and overexpressed (HER2, GRB7, PNMT, MLN64, MGC9753, and MGC1483) (Kauraniemi et al., 2003). The use of genome-wide microarray methods has enabled more comprehensive studies of amplified genomic regions in breast cancer samples. For example, Staaf et al. found the smallest region of amplification in HER2þ cancers to be 85.92 Kbp, including six genes (TCAP, PNMT, PERLD1, HER2, C17orf37 and GRB7) (Staaf et al., 2010). Of the genes co-amplified with HER2, STARD3 and GRB7 were suggested to functionally contribute to proliferation of HER2þ cell lines based on RNA interference studies (Kao and Pollack, 2006). Moreover, GRB7 protein overexpression has been shown to be an independent prognostic factor in breast cancer (Ramsey et al., 2010). Previous studies in breast (Yang et al., 2006) and liver (Zender et al., 2006) cancer also suggest that a single amplicon may harbor two or more cooperating oncogenes. Thus, the identification of co-amplified genes affecting cancer development and drug response would be a useful starting point in the development of new therapeutic strategies for HER2þ breast cancer patients. To characterize the impact of genes in cancer, the concept of oncogene and non-oncogene addiction has recently been coined (Luo et al., 2009). Many genes included in amplicons, even outside the core amplified region, could possibly contribute to the “amplicon addiction” phenotype in individual tumors. Thus, inhibition of such genes could be therapeutically useful.

To systematically study the multiple HER2 amplicon genes and their contribution to the functional phenotype and response to trastuzumab, we have used oligonucleotide aCGH to define the HER2 amplicon break points in 71 HER2þ tumors and 10 HER2þ cell lines. Based on these results, we silenced 23 genes in the amplicon by RNA interference in trastuzumab responding and non-responding HER2þ breast cancer cell lines.

2. Materials and methods

2.1. Cell lines

11 breast cancer cell lines were grown and cultured following recommended conditions. Of these 10 were HER2þ; BT474, HCC202, SKBR3, UACC812, HCC1954 and HCC1569 were obtained from American Type Culture Collection (ATCC, USA), JIMT1 was obtained from German Collection of...
Microorganisms and Cell Cultures (DSMZ, Germany). Both ATCC and the DSMZ authenticate all human cell lines by DNA-typing using short tandem repeats. The DSMZ uses additional cytogenetic and immunophenotypic tests, and ATCC uses amelogenin for gender determination. SUM190 and SUM225 were kindly given by Stephen Ethier from Karmanos Cancer Institute in Michigan USA, whereas KPL4 was kindly provided by J Kurebayashi from Kawasaki Medical School in Japan. The HER2-negative MCF7 cells were obtained from Interlab Cell Line Collection (ICLC, Italy) and used as control. Cells were cultured for a maximum of 30 passages prior to use.

2.2. Primary tumors

Copy number changes from Agilent Human Genome CGH microarray 244K from 71 tumors were used to describe the HER2 amplicon. Data from 54 tumors were retrieved from GEO (GSE17907) (Sircoulomb et al., 2010) and 17 additional tumors from (GSE32291, GSE20394) (Langerod et al., 2007). Using the ASCO guidelines HER2+ for FISH (ratio of HER2 gene signals to chromosome 17 signals of more than 2.2) (Wolff et al., 2007), we defined the tumor to be HER2+ with copy number gain >0.66 (log2 scale). All 71 tumors included had copy number gain >0.9 for HER2.

2.3. Response to trastuzumab

Trastuzumab half maximal effective concentration (EC₅₀) was determined for 10 breast cancer cell lines by exposing cells to trastuzumab in 8 different concentrations ranging from 10 pM to 100 μM in 384-well plates. Cells were treated for 3–5 days, and cell viability was measured using the CellTiter-Glo® assay (CTG, Promega Corp, Madison, WI). Cells with a decrease in cell viability of >20% were defined as responsive to the drug. The maximum drug effect was compared to non-treated samples. Data were analyzed using GraphPad Prism 4 nonlinear regression curve fitting software.

2.4. aCGH analysis

Array-based CGH was carried out using Agilent Human Genome CGH 244K microarray according to the protocol provided by the manufacturer (Agilent Technologies, Palo Alto, CA). Genomic DNA pooled from healthy female donors was used as reference in all hybridizations. Briefly, one μg of digested and purified tumor and reference DNA was labeled with Cy5-dUTP and Cy3-dUTP (Perkin–Elmer, Wellesley, MA), respectively, in a random priming reaction using BioPrime DNA Labeling System (Invitrogen, Carlsbad, CA). Labeled tumor and reference samples were pooled and hybridized onto the arrays according to the protocol. After hybridization arrays were washed and scanned with a laser scanner (G2565 Scanner, Agilent Technologies, Carlsbad, CA). The expression of cPARP (Abcam ab32064), Ki67 (Dako M7240), HER2 (Dako A0485), phospho-AKT (Cell Signaling Technology, Beverly, MA, USA), phospho-p70-S6K (Cell Signaling Technology) and p27 (Cell Signaling Technology) were detected by staining the slides with antibodies, followed by exposure to Alexa Fluor 680-tagged and IRDye 800 CW-tagged secondary antibodies (Invitrogen Inc., Carlsbad, CA, USA and Rockland immunocchemicals, respectively). To normalize the signal to total protein amount, the arrays were stained with SYPRO® Ruby protein Blot stain (Invitrogen Inc., Carlsbad, CA). The slides were scanned with a Tecan LS400 (Tecan Inc., Durham, NC, USA) microarray scanner and an Odyssey LI-COR IR-scanner (LI-COR Biosciences, Lincoln, NE, USA) to detect total protein and the specific antibody signals, respectively. Array-Pro Analyzer microarray analysis software (Median Cybernetics Inc., Bethesda, MD, USA) was used for analyzing data. Data were Z-score normalized. The LMA assay raw data from co-treatments were divided by the corresponding total protein staining (SYPRO®).
Ruby protein Blot stain), log2 transformed and Z-score standardized to the medians of non-treated wells.

### 2.7. Cell cycle analysis

Cell cycle analyses were carried out from nuclear preparations. Cells (60,000–80,000 per well on 24-well plates) were transfected with siRNA and incubated for 48 h. Thereafter, the cells were trypsinized, processed for cell cycle analysis with a BD CycleTest Plus DNA reagent kit (BD Biosciences, San Jose, CA, USA) and examined for cell cycle distribution using FACSArray (BD Biosciences). Data were analyzed using Multicycle software (Phoenix FlowSystems, San Diego, CA, USA).

### 2.8. Validation of siRNA knock-down efficacy

Efficacy of HER2, PPP1R1B, GRB7, STARD3, PERLD1, and PSMD3 single siRNAs and two siRNAs for the same gene in combination was validated by quantitative RT-PCR using TaqMan in SKBR3 and KPL4 cells. Cells only and Scramble siRNA (Qiagen) were used as controls. In short, total cellular RNAs were isolated with RNeasy RNA isolation kit (Qiagen). For cDNA synthesis, 100 ng of total RNA was reverse transcribed with a High Capacity DNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Thereafter, the cDNAs were diluted 1/10 and TaqMan quantitative real-time-PCR analysis was carried out with an Applied Biosystems 7900HT instrument using specific primers for the amplicon genes and beta-actin designed by the Universal ProbeLibrary Assay Design Center (Roche Applied Biosciences, Basel, Switzerland). The sequences of the primers were as follows (forward, reverse): HER2 (gggaaacctggaactcctc, ccctgcacctcctggata), PERLD1 (cacaactgctcctgcta, cggctcttgaaagaacagga), GRB7 (ggaacgggtgtcctgctac, gcagctcttgaaagaacagga), PPP1R1B (ccaccacccgctgga, gaagctcttgaaagaacagga), and beta-actin (ccacccgctgga, gaagctcttgaaagaacagga). The fluorescent TaqMan probes were obtained from Roche Human Probe Library. The results were analyzed with SDS 2.3 and RQ manager software (Applied Biosystems), and the expression of mRNAs was determined by the relative quantitation method using Beta-actin as an endogenous control. Data were collected from two separate biological experiments, which were both run in triplicates.

### 3. Results

#### 3.1. Response to trastuzumab

The half maximal effective concentration (EC<sub>50</sub>) of trastuzumab was determined for 9 HER2+ breast cancer cell lines. SKBR3 and BT474 were trastuzumab sensitive with an EC<sub>50</sub> of 1.02 nM ± 0.71 nM (26.7% decrease in cell viability) and 0.98 nM ± 0.08 nM (34.3% decrease in cell viability), respectively. KPL4, JIMT1, HCC1954, HCC1569, HCC202 and SUM225 did not show a response to trastuzumab (range: 2–18% decrease in cell viability), and were thus considered drug resistant. SUM190 showed a 22.3% response to trastuzumab, but with an EC<sub>50</sub> of 0.67 mM ± 0.6 mM and was thus also considered trastuzumab resistant (Table 1).

#### 3.2. The size of the HER2 amplicon

We used copy number changes from Agilent 244K arrays to study the size of the HER2 amplicon in detail in tumors and cell lines. The smallest common region of amplification found in all of the 71 tumors analyzed was 78.61 Kbp, including six genes; STARD3, TCAP, PNMT, PERLD1, HER2, and C17orf37. Ninety percent (64/71) of the tumors shared a 255.74 Kbp amplification region consisting of ten genes; NEUROD2, PPP1R1B, STARD3, TCAP, PNMT, PERLD1, HER2, C17orf37, GRB7 and ZNFN1A3. Sixty percent (43/71) of the tumors had a 928.93 Kbp common amplification with 27 genes, delimited by RPL19 and NR1D1 on the centromeric and telomeric side respectively. The average size of the amplicon in the tumors was 1.74 Mbp (range: 0.31 Mbp–13.60 Mbp). The cell lines showed an amplification window similar to the tumors, with breakpoints mimicking those seen in the tumors (Figure 1). The average size of the amplicon seen in the cell lines was 1.47 Mbp (range: 0.37 Mbp–3.29 Mbp). The trastuzumab responding and non-responding cell lines showed a similar amplification window, and the copy number change levels were in fact higher in the non-responsive than in the responsive cell lines (mean log2: 4.59 and 3.54 respectively).

#### 3.3. Silencing the amplicon genes

Five cell lines, two trastuzumab responsive; BT474 and SKBR3, two trastuzumab non-responsive; JIMT1 and KPL4, and MCF7 as a control, were subjected to siRNA screening in 384-well plates with cell viability as an endpoint. siRNAs were available as a control, were subjected to siRNA screening in 384-well plates with cell viability as an endpoint. siRNAs were available for 23 of the 27 genes seen amplified in over 60% of the tumors, and 2–4 siRNAs per gene were screened (Supplementary Table 1). In order to look at several endpoints at the same time, additional siRNA screens in 384-well plates were performed and samples were prepared for protein lysate microarrays (LMA) after 72-h incubation. The lysates were printed onto glass slides, and stained with HER2, cPARP, Ki67, phospho-AKT, phospho-p70-S6K, and p27 antibodies (Supplementary Table 2).

### Table 1 — Determination of trastuzumab response of the cell lines used. Half maximal effective concentration (EC<sub>50</sub>) and response to trastuzumab (% decrease in cell viability, CellTiter-Glo®) were determined either after 3 days (3d) or 5 days (5d).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Effect (%) 3d/5d</th>
<th>EC50 3d/5d</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT474</td>
<td>13.5/34.3</td>
<td>–/–9.8E–10</td>
</tr>
<tr>
<td>HCC1569</td>
<td>13/13</td>
<td>3.2E–09/6.3E–07</td>
</tr>
<tr>
<td>HCC1954</td>
<td>9.9/9.9</td>
<td>–/–4.1E–05</td>
</tr>
<tr>
<td>HCC202</td>
<td>6.9/2.0</td>
<td>–/–</td>
</tr>
<tr>
<td>JIMT1</td>
<td>6.6/3.1</td>
<td>5.3E–06/1.3E–08</td>
</tr>
<tr>
<td>KPL4</td>
<td>14.5/N/A</td>
<td>–/–N/A</td>
</tr>
<tr>
<td>MCF7</td>
<td>N/A/N/A</td>
<td>N/A/N/A</td>
</tr>
<tr>
<td>SKBR3</td>
<td>26.7/N/A</td>
<td>1E–09/N/A</td>
</tr>
<tr>
<td>SUM190</td>
<td>22.3/15.5</td>
<td>6.7E–04/6.2E–05</td>
</tr>
<tr>
<td>SUM225</td>
<td>18.0/10.3</td>
<td>1.1E–04/–</td>
</tr>
<tr>
<td>UACC2812</td>
<td>N/A/N/A</td>
<td>N/A/N/A</td>
</tr>
</tbody>
</table>
Antibodies were selected based on their importance in breast cancer and HER2 + pathways. HER2 knock-down led to a significantly decreased cell viability measured by CTG in BT474 and SKBR3, whereas less or no effect was seen in JIMT1 and KPL4 (Figure 2A). Likewise, cell cycle analysis by FACS revealed that siHER2 induced a significantly larger G1 arrest in the trastuzumab sensitive than resistant cell lines. Cell survival signaling regardless of trastuzumab response. The trastuzumab-resistant cell lines showed reduced cell cycle regulation (staining with p27) when PPARG (peroxisome proliferator activated binding protein) was silenced. Knock-down with the individual siRNAs and their combination was validated for HER2 (Figure 2D), GRB7, STARD3, PERLD1, PSMD3 and PPP1R1B (Supplementary Figure 2) genes using TaqMan. A knock-down of ~80% compared to control cells was seen in SKBR3 and KPL4, indicating a similar knock-down in the responsive and the non-responsive cell lines.

### 3.4 Knock-down of two HER2 amplicon targets at the same time

We selected PPP1R1B, STARD3, PERLD1, GRB7, ZPPB2, GSDM1, PSMD3 and THRA for further testing based on their co-amplification with HER2 and their biological functions. We hypothesized that the co-amplification with HER2 leads to a dependency on several genes, and we therefore silenced these genes one at the time together with siHER2 in BT474, KPL4 and JIMT1 (Supplementary Tables 3 and 4). In addition co-treatment was carried out with trastuzumab and Lapatinib and the combination of these. Indeed, silencing of STARD3 together with HER2 led to a clear additive inhibition of cell viability and increased apoptosis in BT474 (Figure 4A and B). Additive inhibition of cell viability and increased cell death (non-additive) is also observed in KPL4 (Figure 4E and F). Combinatorial treatment with siSTARD3 and Lapatinib gave decreased cell viability in both BT474 and KPL4 compared to the siRNA and drug alone (Figure 4B and F). In JIMT1, siSTARD3 caused inhibition of cell viability, cell proliferation (Ki67) and AKT phosphorylation as well as increased apoptosis (cPARP) (Figure 4C and D). Knock-down of STARD3 (StAR-related lipid transfer (START) domain containing 3) was very efficiently downregulating cell growth in the cell lines tested. Additive effect was also seen with siGRB7 together with inhibition of HER2 in all of the cell lines tested (Supplementary Figure 3). Simultaneous silencing of siGRB7 and siHER2 led to additive effect in both KPL4 and JIMT1. siGRB7 together with Lapatinib led to decreased cell viability and inhibition of pAKT in KPL4. siGRB7 and drug combinations lead to increased apoptosis in BT474 as compared to siRNA or drug treatment alone. Combinatorial treatment with siPERLD1 and Lapatinib show more effect than either treatment alone in KPL4 measured by cell viability and proliferation (Ki67 staining) and pAKT inhibition. siPERLD1 and drug treatments show induced apoptosis in BT474. Combined siPSMD3 and HER2 inhibitions are more efficient than either treatment alone in KPL4, measured by cell viability and proliferation. Silencing of PSMBD3 alone leads to induced apoptosis in KPL4, however, combinatorial treatments with siPSMD3 and HER2 inhibiton by siRNA...
and drugs show additive effect in the same cells at both apoptosis and pAKT inhibition.

Several combinatorial treatments lead to induced apoptosis measured by cPARP, especially in KPL4 cells. Induced apoptosis is seen when silencing PPP1R1B together with HER2 inhibition in KPL4, whereas silencing THRA and GSMD1 together with HER2 inhibition are additive in both BT474 and KPL4. AKT pathway activity measured by phospho-AKT is inhibited in KPL4 by Lapatinib treatment alone. KPL4 cells seem to be more sensitive to EGFR inhibition as Lapatinib treatments and combinatorial treatments with trastuzumab and Lapatinib were in general much more efficient than siHER2 or trastuzumab.

4. Discussion

The concept of oncogene and non-oncogene addiction postulates that many genes included in amplicons in individual tumors may contribute to the “amplicon addiction” phenotype, even if they do not lie in the minimal common region of amplification across all tumors. Hence their joint inhibition could be therapeutically useful. As new technologies using siRNA-based therapies are evolving, genes do not necessary need to be chemically druggable to be clinically important.

In the present study, we studied the genes co-amplified with HER2 at 17q12, in order to reveal their possible role in breast cancer and to search for mechanisms related to trastuzumab resistance. By aCGH analysis, we identified a minimal common region of amplification (MCR) including 6 genes covering 78.61 Kb (STARD3, TCAP, PNMT, PERLD1, HER2, and C17orf37). This agrees with previous studies (Staaf et al., 2010). Importantly, our results show that nearly all tumors amplify a considerably larger region, 1.74 Mbp on average, suggesting that many additional genes in the region around HER2 are also usually amplified and may contribute to the cancer phenotype. Genes around high-level amplicons in breast cancer, such as at 17q12, also frequently undergo rearrangements, such as fusion gene formation (Edgren et al., 2011).
pointing to another mechanism, which may contribute to the importance of genes in the HER2 amplicon.

The ten HER2+ cell lines profiled here showed a similar amplification pattern as the tumors, indicating that these are good models for the in vivo setting. The possible links between amplicon size and trastuzumab resistance in clinical tumors have not received much attention. Our cell line data-set shows no link between amplicon size and trastuzumab response. The amplification magnitude is higher in the non-responsive cell lines, but this finding should be confirmed in additional model systems. However, Bates et al. also reported that subpopulation of HER2+ cancers with very high HER2 expression may be resistant to trastuzumab (Bates et al., 2011).

High copy number gain of HER2 has also been linked to poor prognosis in clinical tumors that are not treated with trastuzumab (Staaf et al., 2010). As about half of the patients do not respond to or will develop resistance to trastuzumab, we chose to functionally study the genes co-amplified in over 60% of the 71 tumors studied, in order to identify alternative or additional therapeutic targets (Figure 1). RNAi silencing of these genes in cell lines revealed that several of the genes in the amplicon are needed for the survival of the HER2 amplified breast cancer cells. Interestingly, HER2 itself has a different role in the trastuzumab responding and non-responding cell lines. siRNA for HER2 leads to a statistically significantly decreased cell viability, as well as cell cycle arrest, in the drug responding cell lines compared to both the drug resistant cell lines and the control cell line MCF7 (Figure 2). This indicates that these cells have activated other pathways to circumvent the inhibition of HER2, in effect becoming independent of HER2. Results from the protein lysate microarrays of siRNA transfected cell lines revealed that silencing of STARD3 led to a greater induction of apoptosis in the non-responsive cell lines compared to the responsive cell lines, implying that STARD3 could be essential for cancer cell survival in these cells. We saw induced apoptosis and inhibition of phospho-AKT by silencing NR1D1 in JIMT1 and to some extent in the SKBR3 cells. NR1D1 as well as PPARBP have been found to up-regulate several genes in the fatty acid synthesis network contributing to abnormal cellular energy metabolism, and to play an important role in HER2+ cancers. Silencing of either gene has previously been reported to decrease the survival of BT474, measured by both apoptosis and cell viability (Kourtidis et al., 2010). Silencing of PPARBP did not show any effect on BT474 in our hands, but we did see evidence of cell cycle arrest (staining with p27) in the drug non-responsive cell lines (KPL4 and JIMT1) suggesting that PPARBP through its regulation of fatty acids synthesis could play some role in sustaining cell growth in some of the trastuzumab-resistant cell lines.

We also tested whether we could find an additive or synergistic effect on the cancer cells by simultaneously inhibiting HER2 together with other genes. Interestingly, we found that silencing of STARD3, GRB7, PSMD3 or PERLD1 together with HER2 lead to an additive inhibition of cell viability, proliferation, AKT pathway activity as well as induced apoptosis when compared to targeting either gene alone (Figure 4, Supplementary Figure 3). This indicates that these genes may be important in the HER2+ cancers and through regulation of cell proliferation, survival and apoptosis pathways. Knock-down of STARD3 was highly efficiently downregulating cell growth and inducing apoptosis in the cell lines, and the
Figure 4 — siSTARD3 effectively inhibits growth of breast cancer cells. Silencing of STARD3 together with HER2 inhibition leads to additive inhibition of cell viability (CTG) and increased apoptosis BT474 (A + B). siSTARD3 causes inhibition of cell viability (C), cell proliferation (Ki67) and AKT phosphorylation as well as increased apoptosis (cPARP) in JIMT1 (D). Additive inhibition of cell viability (E) and increased cell death (F, non-additive) is also observed in KPL4. Statistically significant additive effects are shown, "**" show difference to corresponding controls and "o" to non-co-treated siRNA. For JIMT1 all siSTARD3 treatments, except "Ki67, STARD3 + siHER2", are significantly different from corresponding controls (C + D), this is the case also in KPL4 (F, cPARP), (not shown by * for clarity). Ns = non-significant, */o = p < 0.05, **/oo = p < 0.01, */ooo = p < 0.001.
additive effect when silencing STARD3 together with HER2 inhibition were significant in both trastuzumab responsive and non-responsive cell lines. Overexpression of the GRB7 protein has shown to be an independent prognostic factor in breast cancer (Ramsey et al., 2010). Although we do not find inhibition of GRB7 alone to be efficient in the cell lines tested, the combinatorial inhibition of GRB7 and HER2 show a strong additive effect in several of the cell lines. Previous studies have found that PPP1R1B (t-DARPP-32) is overexpressed in a trastuzumab-resistant BT474 cell line variant. Overexpression of t-DARPP-32 was associated with AKT activation (Belkhiri et al., 2008; Hamel et al., 2010), and down-regulation of t-DARPP-32 led to decrease in AKT phosphorylation. We did not observe down-regulation of phospho-AKT by silencing of PPP1R1B alone, but we did see some additive effects when silencing PPP1R1B and HER2 together. The synergistic effect observed when inhibiting HER2 together with other genes in the amplicon may be explained by the genes having partly overlapping downstream targets, such as the AKT pathway. This also fits with the observations that inhibitors of PI3K signaling pathway have a promising impact on the trastuzumab-resistant cells (Miller et al., 2009; Weigelt et al., 2011), as well as combinatorial treatment in cancer patients (Nahta and O’Regan, 2010). In this scenario, silencing of one gene is, partly or fully, compensated by the activity of the other, with simultaneous inhibition of e.g. both HER2 and STARD3 required for full downstream target inhibition. However, this still does not fully explain why the trastuzumab- and siHER2-resistant cell lines JIMT1 and KPL4 show the same additive effects when HER2 is knocked down together with the other genes. This suggests that the resistant cells are still to some extent dependent on the presence of the HER2 protein although it is no longer the only driver oncogene.

5. Conclusions

Our results suggest that the cells are not only dependent on the primary oncogene in the amplicon, HER2, but also on genes that are responsible for cellular processes that may not be directly oncogenic, but that have become necessary for the oncogenic state. This is in-line with the concept of non-oncogene addiction in cancer cells (Luo et al., 2009). For HER2+ cancers that do not respond to trastuzumab, targeting only HER2 is not sufficient, but combinatorial treatment targeting other non-oncogenes may be necessary to kill the cancer cells. Therefore there is an unmet clinical need to develop and design combinatorial strategies to treat HER+ breast cancers. Results from our siRNA screens indicate that signaling from HER2 and many other gene products from the HER2 amplicon converge on critical cellular pathways, regulating cell proliferation and survival.

Authors’ contributions

KKS planned the experiments, participated in siRNA screens, did the bioinformatical analysis of the siRNA screens and drafted the manuscript. VH performed the siRNA screens and validations, participated in the analysis and drafted the manuscript. HE planned the experiments, participated in the analysis and drafted the manuscript. RM did the lysate microarray experiments. KH participated in siRNA screens. EUD performed aCGH of the tumor material. HKMV performed aCGH on tumor material. NS participated in the siRNA screens and did the siRNA library management. MW performed aCGH on the cell lines. ALBD helped with the scientific interpretation of the data, helped to draft the manuscript. MP drafted the project, helped with the scientific interpretation of the data, helped to draft the manuscript. OK drafted the project, helped with the scientific interpretation of the data, helped to draft the manuscript. All authors read and approved the manuscript.

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Appendix A.
Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2012.10.012.

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