BCAR4 induces antioestrogen resistance but sensitises breast cancer to lapatinib

MFE Godinho1, JD Wulfkuhle2, MP Look3,4, AM Sieuwerts3,4, S Sleijfer3,4, JA Foekens3,4, EF Petricoin III2, LCJ Dorssers1 and T van Agthoven1,4

1Department of Pathology, Josephine Nefkens Institute, Erasmus MC-University Medical Center Rotterdam, Room Be 432A, PO Box 2040, Rotterdam 3000 CA, The Netherlands; 2Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA 20110, USA; 3Department of Medical Oncology, Josephine Nefkens Institute, Erasmus MC-University Medical Center Rotterdam, Rotterdam 3000 CA, The Netherlands; 4Cancer Genomics Center, Josephine Nefkens Institute, Erasmus MC-University Medical Center Rotterdam, Rotterdam 3000 CA, The Netherlands

BACKGROUND: High BCAR4 and ERBB2 mRNA levels in primary breast cancer associate with tamoxifen resistance and poor patient outcome. We determined whether BCAR4 expression sensitises breast cancer cells to lapatinib, and identifies a subgroup of patients who possibly may benefit from ERBB2-targeted therapies despite having tumours with low ERBB2 expression.

METHODS: Proliferation assays were applied to determine the effect of BCAR4 expression on lapatinib treatment. Changes in cell signalling were quantified with reverse-phase protein microarrays. Quantitative reverse-transcriptase polymerase chain reaction (RT–PCR) of ERBB2 and BCAR4 was performed in 1418 primary breast cancers. Combined BCAR4 and ERBB2 mRNA levels were evaluated for association with progression-free survival (PFS) in 293 oestrogen receptor-α (ER)-positive patients receiving tamoxifen as first-line monotherapy for recurrent disease.

RESULTS: BCAR4 expression strongly sensitised ZR-75-1 and MCF7 breast cancer cells to the combination of lapatinib and antioestrogens. Lapatinib interfered with phosphorylation of ERBB2 and its downstream mediators AKT, FAK, SHC, STAT5, and STAT6. Reverse transcriptase–PCR analysis showed that 27.6% of the breast cancers were positive for BCAR4 and 22% expressed also low levels of ERBB2. The clinical significance of combining BCAR4 and ERBB2 mRNA status was underscored by the finding that the group of patients having BCAR4-positive/ERBB2-low-expressing cancers had a shorter PFS on tamoxifen treatment than the BCAR4-negative group.

CONCLUSION: This study shows that BCAR4 expression identifies a subgroup of ER-positive breast cancer patients without overexpression of ERBB2 who have a poor outcome and might benefit from combined ERBB2-targeted and antioestrogen therapy.

British Journal of Cancer (2012) 107, 947–955. doi:10.1038/bjc.2012.351 www.bjcancer.com

Keywords: BCAR4; ERBB2; targeted therapy; breast cancer; tamoxifen resistance

Tamoxifen has an important role in the treatment of patients with oestrogen receptor-α (ER)-positive primary breast cancer, both in the adjuvant and metastatic setting (Davies et al, 2011). Its efficacy is limited by primary (intrinsic) or secondary (acquired) resistance. A better understanding of the mechanisms involved is required to overcome resistance and for developing more effective therapies. Several genes and mechanism causing antioestrogen resistance were identified (Dorssers and Veldscholte, 1997; Van Agthoven et al, 1998; Brinkman et al, 2000; Massarweh and Schiff, 2007; Riggins et al, 2001; Musgrove and Sutherland, 2009; Barone et al, 2010; Van Agthoven et al, 2010), including the novel breast cancer antioestrogen resistance 4 (BCAR4) gene (Meijer et al, 2006). Ectopic expression of BCAR4 causes antioestrogen resistance, anchorage independence, and tumour growth in nude mice (Meijer et al, 2006; Godinho et al, 2011). BCAR4 mRNA is detected in 22–29% of primary breast cancers. High levels are associated with shorter progression-free survival (PFS) in patients treated with tamoxifen for recurrent disease, and associate with poor metastasis-free survival (MFS) and overall survival (OS), reflecting tumour aggressiveness (Godinho et al, 2010).

BCAR4 has been found in several mammalian species, being well conserved in higher primates (Meijer et al, 2006; Godinho et al, 2011). In the functional screening for genes causing tamoxifen resistance, it was isolated from a human placenta cDNA library only (Meijer et al, 2006; Godinho et al, 2011). Searches in public expression databases and in the literature showed that high BCAR4 expression is only found in placenta and the oocyte (Meijer et al, 2006; Godinho et al, 2011). In other normal adult tissues, expression of BCAR4 was not found. The species and tissue-specific expression strongly indicates a role for BCAR4 in mammalian early development and pregnancy. Surprisingly, the BCAR4 gene is absent in the mouse and rat (Godinho et al, 2011). Important differences exist between human and mouse placental development and function. In the mouse, in contrast to the human situation, trophoblast implantation is superficial, the transformation of the uterine arteries depends on maternal factors, and mouse placenta produces fewer placental hormones (Malassine et al, 2003; Carter, 2007). At this point it is only possible to speculate on the function of this gene, but it is likely that differences in placental development and function could explain the
absence in these organisms. BCAR4 may have a function in placenta and early development, therefore it cannot be excluded that in mouse and rat its function has been taken over by other genes.

BCAR4-induced tamoxifen resistance depends on the presence of ERBB2 (HER2) and ERBB3 receptors (Godinho et al, 2010). We hypothesised that BCAR4 expression may sensitise breast cancer cells to the small-molecule tyrosine kinase activity inhibitor of EGFR and ERBB2. In this study, BCAR4-expressing cells were assessed for their sensitivity to lapatinib, given alone and in combination with antioestrogens. In addition, the effects of treatment on ERBB2 and ERBB3 downstream signalling were measured. As increased ERBB2 activity has been associated with resistance to cytotoxic agents in breast cancer, the impact of BCAR4 expression on sensitivity to several cytotoxic drugs was assessed. The results of our cell line studies showed that ectopic expression of BCAR4 results in activation of the ERBB2 signalling pathway without overexpression of ERBB2. Therefore, we determined the incidence of breast cancers expressing BCAR4 and low ERBB2 levels, and how this group of patients fares when treated with tamoxifen for advanced disease.

MATERIALS AND METHODS

Cell lines and culture conditions

ZR-75-1 and MCF7 cell lines were kind gifts of RJB King (ICRF, London) and RB Dickson (NCI, Bethesda), respectively. Cell lines were initially authenticated by karyotyping, and in November 2011 using the AmpFISTR Identifier Direct PCR Amplification Kit (Applied Biosystems International, Nieuwerkerk a/d Ijssel, The Netherlands). Cell lines derived from the breast cancer cell line ZR-75-1 containing empty vector, or expression constructs with BCAR4 (Meijer et al, 2006), BCAR1 (Brinkman et al, 2000), BCAR3 (Van Agthoven et al, 1998), or EGFR (Van Agthoven et al, 1992), and MCF7 breast cancer cells with a construct containing BCAR4 were cultured as previously described (Van Agthoven et al, 1998).

Drug sensitivity assays

Cells were seeded in 96-well plates at a density of 5000 cells per well in 100 µl RPMI 1640 medium (Invitrogen, Breda, The Netherlands). After 24 h, serial dilutions of lapatinib (GlaxoSmithKline, Stevenage, UK), doxorubicin (Pharmachemie B.V., Haarlem, The Netherlands), 5-fluorouracil (Ebewe Pharma, Unterach, Austria), methotrexate (Emthexate PF, Pharmachemie B.V.), ifosfamide (Holoxan, Baxter B.V., Utrecht, The Netherlands), or paclitaxel (Paclitaxel, Ebewe Pharma) were added. All drugs were tested in combination with 17 β-oestriol or 4-hydroxytamoxifen (Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) or ICI182,780 (Zeneca Pharmaceuticals, Macclesfield, UK). To assay the effects of oestrogen, cells were seeded at a density of 5000 cells per well in 100 µl RPMI 1640 without phenol red, supplemented with 6% heat-inactivated bovine calf serum (Hyclone, Logan, UT, USA). Twenty-four hours after seeding, 100 µl of medium containing 0.01, 0.1, or 1 µM lapatinib and increasing concentrations of oestriol were added. WST-1 proliferation assays (Roche Diagnostics, Almere, The Netherlands) were performed on ZR-75-1- or MC7-derived cell lines after 5 or 6 days, respectively. IC50 values were estimated by sigmoid inhibitory effect models 107 and 108 as implemented in the software programme Phoenix WinNonLin 6.1 (Pharsight, Mountain View, CA, USA).

Inhibition of gene expression by small interfering (si)RNAs

Transfections with HiPerfect (Qiagen, Venlo, The Netherlands) were performed according to the manufacturer’s instructions. Small interfering RNAs were On TARGETplus-SMARTpools, each consisting of three different oligonucleotides: EGFR (L-003114-00-0005), ERBB2 (L-003126-00-005), ERBB3 (L-003127-00-0005), and ERBB4 (L003128-00-0005; Dharmacon, Perbio-Science, Etten-Leur, The Netherlands). Final concentration of siRNA was 5 nM. WST-1 assays were performed after 6 days.

Reverse-phase protein microarrays

Cells cultured in oestradiol- or 4-hydroxytamoxifen-containing medium were treated with or without 0.01 or 0.1 µM lapatinib for 17 h. Cells were rinsed with ice-cold DPBS (Gibco, Invitrogen), and lysed with pre-heated (75 °C) extraction buffer consisting of equal parts of T-PER (Pierce, Thermo Scientific, Etten-Leur, The Netherlands) and Tris-Glycine-SDS Sample Buffer (Invitrogen) containing PhosSTOP Phosphatase Inhibitors, Complete Mini Protease Inhibitors (Roche Diagnostics), and 4% of β-mercaptoethanol (Merck, Schiphol-Rijk, The Netherlands). Lysates were boiled for 8 min and stored at −80 °C. Reverse-phase protein microarray analysis was performed as described (Van Agthoven et al, 2012). A list of antibodies used is presented in Supplementary Table 1.

Patient samples

ERBB2 and BCAR4 mRNA levels were measured in 1418 ER-positive and negative primary breast cancers as described in Van Agthoven et al (2009) and Godinho et al (2010). Here we assessed the prognostic and predictive values of a combined BCAR4 and ERBB2 status. BCAR4 and ERBB2 were determined according to the definitions/cut points in the aforementioned studies. To determine the association of the combination of BCAR4 and ERBB2 mRNA levels and DFS, 293 samples from patients with ER-positive cancers who received tamoxifen treatment as first-line therapy for metastatic disease were analysed. The associations of the combined BCAR4 and ERBB2 levels with tumour aggressiveness in terms of MFS and OS were determined on 497 ER-positive cancers from patients with lymph node–negative disease. None received systemic adjuvant therapy. Statistical analyses were performed as previously detailed (Godinho et al, 2010).

Quantification of gene expression

RNA isolation of cell lines, complementary DNA synthesis, normalisation to reference genes and quantification were performed as described (Sieuwerts et al, 2005; Van Agthoven et al, 2009; Godinho et al, 2010). TaqMan gene expression assays for EGFR-Hs00176538_m1, ERBB2-Hs00706901_m1, ERBB3-Hs00176538_m1, ERBB4-Hs00171783_m1, and BCAR4-Hs00415922_m1 were used according to the recommendations of the supplier Applied Biosystems International.

RESULTS

Inhibition of ERBB2/3 expression abrogates BCAR4-induced antioestrogen-resistant proliferation

Previously we have shown that BCAR4-induced tamoxifen-resistant proliferation of ZR-75-1 cells depends on the presence of ERBB2 and ERBB3 (Godinho et al, 2010), while ERBB2 is not overexpressed or amplified in this cell line (Hollestelle et al, 2010). In MCF7 cells, we investigated whether BCAR4 expression also induces ERBB2/3-mediated proliferation. The expression of the four ERBB receptors were inhibited with siRNAs, in the absence or presence of the pure antioestrogen IC182,780. In contrast to 4-hydroxytamoxifen, this antioestrogen fully inhibits growth of wild-type MCF7 cells. Inhibition of mRNA transcripts was verified by quantitative real-time reverse transcriptase polymerase chain
reaction (RT–PCR, and was more than 70% for EGFR, 88% for ERBB2, 66% for ERBB3, and 75% for ERBB4.

In foetal bovine serum-containing medium, the proliferation capacity of MCF7 cells expressing BCAR4 (MCF7/BCAR4) and MCF7/vector containing cells was not affected by the inhibition of the expression of the ERBB receptors (Figure 1A and B). Similarly to BCAR4 expression in ZR-75-1 cells, MCF7/BCAR4 cells were antioestrogen resistant and able to grow in the presence of ICI182,780 (Figure 1A). Under this culture condition, the inhibition of ERBB2, ERBB3, and ERBB4 expression resulted in decreased cell proliferation, indicating that also in MCF7/BCAR4 cells, ERBB signalling is involved in antioestrogen resistance. Growth of MCF7/vector cells was fully inhibited by ICI182,780, and inhibition of the ERBB receptor expression had no further effect (Figure 1B).

BCAR4 expression increases the sensitivity of cells to lapatinib

We speculated that BCAR4 expression may increase the sensitivity to the EGFR/ERBB2 tyrosine kinase inhibitor lapatinib. Sensitivity to lapatinib was determined in ZR-75-1 cells containing empty expression vector (ZR/vector) or BCAR4 (ZR/BCAR4). Cells expressing BCAR1 (ZR/BCAR1), BCAR3 (ZR/BCAR3), or EGFR (ZR/EGFR) were used for comparison. These latter genes were shown to induce tamoxifen resistance by mechanisms independent of ERBB2 and ERBB3 (Van Agthoven et al, 1992, 1998, Brinkman et al, 2000; Meijer et al, 2006). Titration experiments showed that cells expressing BCAR4 were the most sensitive to lapatinib in the presence of oestradiol (Figure 2A). The IC50 values for ZR/BCAR4 cells were 10- to 20-times lower than the IC50 determined for the other cell lines. In the presence of oestradiol and lapatinib expression of BCAR1, BCAR3, or EGFR had no impact on proliferation, which was similar to the empty vector-containing cells.

Lapatinib sensitivity was also determined in MCF7 cells. In the presence of fetal bovine serum alone, the determined IC50 values for MCF7/BCAR4 cells were similar to the IC50 values determined for MCF/vector cells (7–9 μM and 8–12 μM, respectively). This is in agreement with the inhibition of the ERBB receptors having no effect on proliferation of MCF7/BCAR4 under this culture condition (Figure 1). Under these culture conditions, the cells apparently depend on the ER pathway for proliferation.

Antioestrogens enhance the sensitivity of BCAR4-expressing cells to lapatinib

We tested whether antioestrogens could enhance the sensitivity to lapatinib. Proliferation of wild-type ZR-75-1 cells is fully inhibited by 1 μM 4-hydroxytamoxifen in the culture medium. ZR/BCAR4 was also the most sensitive cell line to the combination of lapatinib and 4-hydroxytamoxifen compared with ZR/BCAR1, ZR/BCAR3, or ZR/EGFR cells (Figure 2B). Tamoxifen further increased the sensitivity of ZR/BCAR4 cells to lapatinib by approximately...
three-fold. A very similar lapatinib dose–response curve was obtained with the presence of ICI182,780 (data not shown). ZR-75-1 cells with forced expression of the EGFR are tamoxifen resistant and oestrogen-independent in the presence of 10 ng/ml of EGF (Van Agthoven et al, 1992). Compared with oestriadiol-stimulated cultures, ZR/EGFR cells were six-fold more sensitive than controls to the combination of lapatinib, 4-hydroxytamoxifen, and EGF. ZR/BCAR1 and ZR/BCAR3 cells showed similar lapatinib dose–response curves in oestriadiol and 4-hydroxytamoxifen-containing medium, approximately 35-fold less sensitive than ZR/BCAR4 cells. Growth of ZR/vector cells was fully inhibited by 4-hydroxytamoxifen, therefore the sensitivity to lapatinib under this culture condition is not informative (data not shown). ICI182,780 increased the sensitivity of MCF7/BCAR4 cells to lapatinib by approximately 10-fold.

Lapatinib inhibits ERBB2 signalling in BCAR4-expressing cells

Reverse-phase protein microarray analysis was used to determine the effects of lapatinib treatment on the levels of 68 total or phosphorylated proteins having a role in survival, motility, death, growth, metabolism, and inflammation (Supplementary Table 2). To circumvent the problem that changes in phosphorylation were solely due to toxicity, cells were cultured in medium without lapatinib or with low doses of 0.01 or 0.1 μM lapatinib for 17 h. These concentrations resulted in limited growth inhibition after 5 days in culture (Figure 2A and B). Lapatinib treatment had no prominent effects on protein phosphorylation in ZR/vector, ZR/BCAR1, ZR/BCAR3, or ZR/EGFR cells, while clear changes were observed for ZR/BCAR4 cells (Figure 3A). We quantified the effects on the phosphorylation of its target, the ERBB2 receptor, the observed for ZR/BCAR4 cells (Figure 3A). We quantified the effects on the phosphorylation of its target, the ERBB2 receptor, the observed for ZR/BCAR4 cells (Figure 3A). We quantified the effects on the phosphorylation of its target, the ERBB2 receptor, the observed for ZR/BCAR4 cells (Figure 3A). We quantified the effects on the phosphorylation of its target, the ERBB2 receptor, the observed for ZR/BCAR4 cells (Figure 3A).

As a typical example, a dose–response curve of ZR/vector, ZR/BCAR4, ZR/BCAR1, ZR/BCAR3, and ZR/EGFR cells to methotrexate is shown in Supplementary Figure S1. No major differences in sensitivity to the drug between the different cell lines, either in the presence of oestriadiol (Supplementary Figure S2A) or 4-hydroxytamoxifen (Supplementary Figure S2B), were observed. Similar results were obtained for ifosfamide, 5-fluorouracil, doxorubicin, and paclitaxel, indicating no changes in sensitivity to conventional drugs due to the expression of BCAR4. Moreover, no major differences were found between the IC_{50} values determined for all the BCAR cell lines and for the control cells (Supplementary Table 3), with exception of ZR/BCAR1 cells being less sensitive to doxorubicin, and ZR/EGFR cells being less sensitive to doxorubicin and 5-fluorouracil.

BCAR4 mRNA levels may define a subgroup of patients who are eligible for treatment with established ERBB2 inhibitors

At present, only patients with breast cancers overexpressing ERBB2 or with gene amplification are eligible for ERBB2-targeted therapies. Our functional in vitro studies show that BCAR4 activates the ERBB2 pathway yielding resistance against anti-oestrogens in cell lines not overexpressing ERBB2. This could imply that BCAR4 expression identifies an additional subgroup of patients with activated ERBB2, but lacking ERBB2 overexpression. To investigate alterations in drug sensitivity, cells were cultured in oestriadiol- or 4-hydroxytamoxifen-containing medium and increasing concentrations of the different chemotherapeutics. For example, lapatinib completely inhibited ERBB2 phosphorylation in ZR/BCAR4, ZR/BCAR1, ZR/BCAR3, and ZR/EGFR cells to methotrexate is shown in Supplementary Figure S1. No major differences in sensitivity to the drug between the different cell lines, either in the presence of oestriadiol (Supplementary Figure S2A) or 4-hydroxytamoxifen (Supplementary Figure S2B), were observed. Similar results were obtained for ifosfamide, 5-fluorouracil, doxorubicin, and paclitaxel, indicating no changes in sensitivity to conventional drugs due to the expression of BCAR4. Moreover, no major differences were found between the IC_{50} values determined for all the BCAR cell lines and for the control cells (Supplementary Table 3), with exception of ZR/BCAR1 cells being less sensitive to doxorubicin, and ZR/EGFR cells being less sensitive to doxorubicin and 5-fluorouracil.

As several studies indicate an association between ERBB2 overexpression and resistance to chemotherapy (reviewed in Tan and Yü, 2007), and BCAR4 expression enhances ERBB2 signalling, we determined the sensitivity of BCAR4-expressing cells to drugs currently included in common breast cancer treatment regimens. To investigate alterations in drug sensitivity, cells were cultured in oestriadiol- or 4-hydroxytamoxifen-containing medium and increasing concentrations of the different chemotherapeutics. For example, lapatinib completely inhibited ERBB2 phosphorylation in ZR/BCAR4, ZR/BCAR1, ZR/BCAR3, and ZR/EGFR cells to methotrexate is shown in Supplementary Figure S1. No major differences in sensitivity to the drug between the different cell lines, either in the presence of oestriadiol (Supplementary Figure S2A) or 4-hydroxytamoxifen (Supplementary Figure S2B), were observed. Similar results were obtained for ifosfamide, 5-fluorouracil, doxorubicin, and paclitaxel, indicating no changes in sensitivity to conventional drugs due to the expression of BCAR4. Moreover, no major differences were found between the IC_{50} values determined for all the BCAR cell lines and for the control cells (Supplementary Table 3), with exception of ZR/BCAR1 cells being less sensitive to doxorubicin, and ZR/EGFR cells being less sensitive to doxorubicin and 5-fluorouracil.

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Clinical relevance of combined BCAR4 and ERBB2 status

BCAR4 and ERBB2 have been found to be independently predictive for tamoxifen resistance in recurrent breast cancer. While ERBB2 phosphorylation of ERBB2 and downstream targets is inhibited in the presence of oestriadiol and lapatinib. The addition of 4-hydroxytamoxifen increased ERBB2 levels and downstream signalling. Therefore, we hypothesised that if the ERBB2 signalling pathway is inhibited by lapatinib, BCAR4-expressing cells may switch to the ER pathway to sustain survival and proliferation. To test this, we analysed the effects of lapatinib treatment on oestriadiol dependence in short-term cultures. While ZR/BCAR4 cells showed maximal proliferation capacity in the absence of oestriadiol (Figure 4A), ZR/vector cells required supplementation of 10–100 μM of oestriadiol. Figure 4B shows that in the presence of 0.01 or 0.1 μM lapatinib, oestrogen dependence of ZR/vector cells remained unchanged. In contrast, proliferation of ZR/BCAR4 cells was markedly inhibited by lapatinib in the presence of more than 10 μM of oestriadiol (Figure 4A). These results indicate that ZR/BCAR4 cells can evade the growth inhibitory effects of lapatinib in part through ER signalling.
was not associated with the natural course of the disease in untreated lymph node–negative ER-positive patients with primary breast cancer, patients with BCAR4-positive tumours had a shorter MFS and OS compared with BCAR4-negative tumours (Van Aghoven et al., 2009; Godinho et al., 2010).

Here we assessed the associations of combined BCAR4 and ERBB2 status and clinical tamoxifen resistance in recurrent breast cancer. mRNA levels of 293 ER-positive primary cancers of ERBB2 status and clinical tamoxifen resistance in recurrent breast cancer. mRNA levels of 293 ER-positive primary cancers of

with high ERBB2 levels had the shortest PFS, regardless of BCAR4 status (Table 1). The Kaplan–Meier analysis visualises the different outcomes of the patients stratified according to the combined BCAR4 and ERBB2 status (Figure 3B). In the multivariate analysis, the power of the combination of BCAR4 expression and low levels of ERBB2 was independent of the traditional predictive factors for PFS (BCAR4-negative/ERBB2-low vs BCAR4-positive/ERBB2-low, HR = 1.50, P = 0.011; Table 1).

To assess the associations of combined BCAR4 and ERBB2 levels and tumour aggressiveness, we analysed mRNA status in 497 primary breast cancers. All patients had ER-positive, lymph node–negative cancer and did not receive adjuvant systemic therapy, allowing the analysis of the natural course of the disease. The mRNA levels were analysed for association with the end points MFS and OS. Metastasis-free survival in patients with BCAR4-positive/ERBB2-low tumours was not significantly different from patients with BCAR4-negative/ERBB2-low tumours. Patients with
BCAR4-positive/ERBB2-high tumours had the shortest MFS (multivariate HR = 1.95, P = 0.026; Supplementary Table 4). Analysis for OS indicated that patients with BCAR4-positive/ERBB2-low tumours had a significantly shorter OS than patients with BCAR4-negative/ERBB2-low tumours. This difference was independent of the traditional prognostic factors (multivariate HR = 1.54, P = 0.021). Patients with BCAR4-positive/ERBB2-high tumours had the shortest OS from all groups (multivariate HR = 2.25, P = 0.004; Supplementary Table 4).

DISCUSSION

In this study we show that BCAR4 expression sensitises two breast cancer models to lapatinib. As BCAR4 expression in cell lines did not change the sensitivity to different chemotherapeutic agents, the increased sensitivity to lapatinib is not due to a general mechanism of drug resistance. The combination of lapatinib and tamoxifen treatment is more effective at inhibiting breast cancer cell growth than lapatinib alone (Chu et al, 2005; Leary et al, 2010). Also in our BCAR4-expressing cell models, the combination of lapatinib and antioestrogens was more potent in inhibiting cell growth than lapatinib alone; indicating that blocking the ERBB2 pathway with lapatinib re-sensitises BCAR4-expressing cells to antioestrogens. Breast tumours have been shown to alternate between ER and ERBB2 signalling, and inhibition of one of the pathways reactivated the other (Gutierrez et al, 2005; Lipton et al, 2005; Massarweh and Schiff, 2006; Munzone et al, 2006; Creighton et al, 2008). It has also been reported that increased ER signalling occurs in lapatinib-treated breast cancer cell lines (Xia et al, 2006; Leary et al, 2010). In the presence of lapatinib and increasing levels of oestradiol, ZR/BCAR4 cells exhibited comparable cell growth kinetics as oestrogen-dependent parental cells (Figure 5A). In culture medium containing lapatinib without oestradiol growth of ZR/BCAR4 cells was strongly inhibited. This suggests that BCAR4 cells use the ER signalling pathway to survive in the presence of low concentrations of lapatinib. Likewise, MCF7/BCAR4 cells cultured in medium without antioestrogens use the ER pathway to

Figure 4  Lapatinib-treated cells utilise ER signalling for survival. ZR/BCAR4 (A) and ZR/vector (B) cells were plated in the absence (●), 0.01 µM lapatinib (○), 0.1 (■), or 1 µM (□) of lapatinib and different concentrations of oestradiol, as indicated. Results are expressed as a percentage of maximal growth (cultures with 1 nM oestradiol but without lapatinib), as measured with a WST-1 proliferation assay. Average of three replicates and SD values are presented.

Figure 5  Clinical relevance of combined BCAR4 and ERBB2 status. Expression of BCAR4 and ERBB2 mRNA levels was measured in a cohort of primary breast carcinomas. Patients were stratified according to the combined BCAR4 and ERBB2 status as indicated. BCAR4 was divided in negative (no call), and positive low and high by the mean. There is no relation observed between BCAR4 and ERBB2 expression. (A) Prevalence of breast tumours expressing BCAR4 and low levels of ERBB2. Number of tumours and percentages are shown for each group. (B) Progression-free survival of patients with ER-positive breast cancer who received first-line tamoxifen monotherapy for recurrent breast cancer. Patients with ERBB2-high tumours were grouped irrespective of their BCAR4 status. The patients at-risk at 12-month intervals are indicated. Abbreviations: neg = negative, pos = positive.
These cells are antioestrogen-resistant, but similar to ZR/BCAR4 cells, proliferation is reduced when ERBB2 or ERBB3 are knocked-down. This indicates that MCF7/BCAR4 cells are also dependent on the ERBB2 and ERBB3 pathway to overcome the inhibitory effects of antioestrogens. Apparently, BCAR4 expression enables cells to alternate between signalling pathways to escape the inhibition of one of them.

The mechanism by which BCAR4 activates the ERBB2 and ERBB3 receptors is still unknown, but several hypotheses can be considered. BCAR4 may encode a very small protein, and its predicted anchor signal and two transmembrane domains suggest that the protein is located at cell membranes. Because of its possible location and interaction with ERBB receptors, there is a possibility that BCAR4 may be a target for ADAM proteins, which cleave ERBB ligands (Mochizuki, 2007). This way, cleaved BCAR4 would be free to bind ERBB3, activating ERBB2 and ERBB3 signalling. BCAR4 may interact with ERBB2 through the cell membrane, similar to MUC4 (Carraway et al., 2001). A mechanism similar to nucleolin, which interacts intracellular and activates the ERBB receptors (Di Segni et al., 2008). The hypothesis that BCAR4 may be a secreted protein can also not be excluded. Another possibility is that the BCAR4 protein interacts with and stabilises the ERBB2/ERBB3 dimer, or interferes with the internalisation and/or intracellular transport of the receptors. It has been shown that mucins can influence receptor trafficking and localisation, and because of that, can modulate receptor tyrosine kinase signalling (Funes et al., 2006).

Lapatinib treatment has been shown to prevent ubiquitination and degradation of ERBB2, resulting in the accumulation of inactive receptors at the plasma membrane (Scaltriti et al., 2009). Exposure of ZR/BCAR4 cells to lapatinib resulted in modestly increased ERBB2 protein levels. Addition of 4-hydroxytamoxifen to ZR/BCAR4 cells further increased ERBB2 levels, in agreement with earlier observations (Van Agthoven et al., 1994; Bates and Sutherland, 2009; Van Agthoven et al., 2007; Musgrove et al., 2009). In our model, activity of ERBB2, ERBB3, and the downstream mediators studied are efficiently inhibited in medium containing oestradiol and a low dose of lapatinib. However, cell proliferation was only partially inhibited, again suggesting an escape route via the ER signalling pathway.

EGFR and ERBB2 overexpression is well documented as being involved in tamoxifen resistance (Riggins et al., 2007; Musgrove and Sutherland, 2009; Van Aghthoven et al., 2009). EGFR is not involved in our cell models of endocrine resistance because oestrogen-dependent ZR-75-1 and MCF7 cells are devoid of detectable EGFR expression (Van Aghthoven et al., 1992), and ERBB2 is present but not overexpressed nor amplified (Hollestelle et al., 2010). Introduction of BCAR4 activates ERBB2 signalling and induces resistance against antioestrogens (Van Aghthoven et al., 2012). This suggests that not only ERBB2 overexpression or amplification is associated with tamoxifen resistance, but that the mere activation of the receptor may also have a role in the process. This is in agreement with earlier findings that other models of endocrine resistance, LTED and LTAM cells, showed increased activation of ERBB2 and downstream signalling (Leary et al., 2010). In addition, it has been hypothesised that moderate, as well as low ERBB2 levels, may generate a strong mitogenic signal when the receptor is activated by dimerisation with EGFR or ERBB3 (Frogne et al., 2009).

In ZR/BCAR4 cells cultured with 4-hydroxytamoxifen, the IC$_{50}$ for lapatinib was approximately 1 µM. This concentration is achieved in the plasma of patients treated with the recommended daily dose of 1.5 mg (Burris et al., 2005), emphasising the potential feasibility of lapatinib as treatment for tamoxifen resistant breast cancer due to BCAR4 expression. Activated ERBB2 has been found in ER-positive tumours classified as negative for ERBB2 expression according to the standard criteria (Frogne et al., 2009). Moreover, emerging evidence shows that some tumours scoring negative for ERBB2 expression benefit from trastuzumab treatment (Paik et al., 2008; Esteva et al., 2010). At present, treatment with ERBB2-targeted therapies is restricted to patients with breast cancers overexpressing ERBB2. Until now, there are no biomarkers to select patients with ER-positive/ERBB2-negative tumours, which are dependent on ERBB2 signalling (Mayer and Arteaga, 2010), and may benefit from ERBB2-targeted therapies. Although it has been shown that the combination of lapatinib and an aromatase inhibitor is not beneficial for ERBB2-negative, endocrine sensitive or endocrine naive metastatic breast cancer patients (Johnston et al., 2009), this remains to be established for BCAR4-positive tumours.

We have shown that co-expression of BCAR4 and low level of ERBB2 occurs frequently, and that these patients have less benefit from tamoxifen treatment. Although our observations do not prove that the ERBB2 signalling pathway is activated in these tumours, our experimental data suggest that this group might benefit from the combination of lapatinib and antioestrogens. The focus of our future studies will be to determine the phosphorylation status of ERBB2 and downstream mediators on micro tissue...
arrays of a large cohort of breast cancers with known follow-up. Patients with primary tumours with high levels of ERBB2 had the shortest PFS, irrespective of their BCA4 levels. On the basis of our results it will now be highly relevant to establish whether these BCA4-positive/ERBB2-low cancers have indeed an activated ERBB2 signalling pathway.

ACKNOWLEDGEMENTS

We are thankful to GlaxoSmithKline for providing Lapatinib, to Walter Loos for performing the ICG_0 calculations, Johan Boender for performing the siRNA transfections and Leendert Looijenga for critical reading of the manuscript. This study was funded by Erasmus MC MRACE grant 2007.

Conflict of interest

JDW: ownership interest and paid consultant for Theranostics Health, LLC. EFP: ownership interest, member of advisory board and unpaid consultant for Theranostics Health, LLC. The remaining authors declare no conflict of interest.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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