Selective recruitment of breast cancer anti-estrogen resistance genes and relevance for breast cancer progression and tamoxifen therapy response

Ton van Agthoven¹, Anieta M Sieuwerts², Danielle Meijer¹, Marion E Meijer-van Gelder², Thecla L A van Agthoven¹, Roya Sarwari¹, Stefan Sleijfer², John A Foekens²,³ and Lambert C J Dorssers¹

¹Department of Pathology, ²Department of Medical Oncology and ³Cancer Genomics Center, Josephine Nefkens Institute, Erasmus MC – University Medical Center Rotterdam, Be 432, Erasmus MC. PO Box 2040, 3000 CA Rotterdam, The Netherlands
(Correspondence should be addressed to L C J Dorssers; Email: l.dorssers@erasmusmc.nl)

Abstract

Although endocrine treatment of breast cancer is effective and common practice, in advanced disease the development of resistance is nearly inevitable. To get more insight into individual genes that account for resistance against hormonal agents, we have executed functional genetic screens and subsequently evaluated the clinical relevance of several identified genes with respect to tumor aggressiveness and tamoxifen resistance in estrogen receptor-positive patients. Estrogen-dependent human breast cancer cells were transduced with different retroviral cDNA expression libraries and subjected to selective cultures with various anti-estrogens. From a total of 264 resistant cell clones, 132 different genes were recovered by PCR. By applying stringent selection criteria, we identified 15 breast cancer anti-estrogen resistance (BCAR) genes individually yielding resistance. BCAR genes were recovered with differential frequencies for the diverse culture conditions and anti-estrogen drugs. Analysis of the relation of BCAR genes (EIF1, FBXL10, HRAS, NRG1, PDGFRα, PDGFRβ, RAD21, and RAF1) with tamoxifen treatment in patients with advanced disease showed significant association with clinical benefit and progression-free survival for EIF1 and PDGFRα mRNA levels. Furthermore, PDGFRα and HRAS mRNA levels were significantly associated with tumor aggressiveness in lymph node-negative patients who had not received adjuvant systemic therapy. In conclusion, our functional genetic screens showed that BCAR genes differ in their ability to confer resistance towards distinct anti-estrogens. Based on the clinical relevance of several BCAR genes, further studies are warranted to characterize the underlying mechanisms, which may ultimately lead to the development of novel treatments and more individualized management of breast cancer patients.

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Introduction

Estrogens play important roles in the establishment and progression of breast cancer. As a consequence, endocrine treatment modalities have been designed to counter the supportive role of estrogens in breast cancer. In the past decades, the anti-estrogen tamoxifen has become a cornerstone in the treatment of estrogen receptor (ER)-positive breast cancer. Large clinical trials have proven its beneficial effects in the adjuvant treatment of localized disease (Early Breast Cancer Trialists’ Collaborative Group 2005), in the prevention of breast cancer in high risk women (Cuzick et al. 2007), and in the metastatic setting. However, in metastatic disease, ~30–40% of the patients with ERα-positive tumors fail to respond to tamoxifen treatment due to intrinsic or de novo resistance of the tumor. Furthermore, in nearly all patients initially showing a response to tamoxifen, progressive disease...
(PD) will ultimately develop while still on treatment (Jaiyesimi et al. 1995, Jordan 1995). To improve outcomes of hormonal therapy and to attenuate the side effects accompanying tamoxifen therapy such as increased risks of endometrial cancer and thromboembolic events (Early Breast Cancer Trialists’ Collaborative Group 1998), several other anti-estrogens have been developed including selective ER modulators, selective ER down-regulator (Lerner & Jordan 1990, Howell et al. 2004), and aromatase inhibitors, the latter blocking the conversion of androgens into estrogens. In recent years, several of these novel drugs have been shown superior to tamoxifen in randomized clinical trials and, consequently, have replaced tamoxifen in various settings (Howell et al. 2004, Howell 2005, Johnston 2005, Come et al. 2008). However, similar to tamoxifen, the development of resistance to these new drugs seems inevitable. The mechanistic basis for the resistant phenotype is thought to originate from the various aspects of estrogen signaling, the interaction with co-regulators, and the interplay with growth factor signaling (Dorssers et al. 2001, Ali & Coombes 2002, Clarke et al. 2003, Gutierrez et al. 2005, Nicholson et al. 2005, Osborne & Schiff 2005, Riggins et al. 2007, Hurtado et al. 2008, Iorns et al. 2008, Zwart et al. 2009).

Through applying this technique with the breast cancer cell line ZR-75-1, we have previously identified a set of seven BCAR genes conferring resistance to tamoxifen (Meijer et al. 2006). In the present study, we explored whether the use of an additional target cell line, an additional cDNA library, and different selective culture conditions using several anti-estrogens would reveal novel BCAR genes. Furthermore, novel BCAR genes identified through this approach were assessed for their clinical relevance in terms of tamoxifen resistance and tumor aggressiveness.

**Materials and methods**

**Cell lines and transduction experiments**

ZR-75-1 cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated bovine calf serum (standard medium) (Hyclone, Logan, UT, USA) and with 1 nM 17β-estradiol (Sigma–Aldrich) as described previously (Van Agthoven et al. 1992). MCF-7 cells were cultured in RPMI medium supplemented with 10% FCS (Sigma–Aldrich) as described (Van Agthoven et al. 1998). Retroviral transduction experiments were performed using retroviral cDNA expression libraries derived from human placenta, human brain, mouse embryo, and HELA cells, a cervical cancer cell line (Clontech). Cell-free supernatants containing viral particles were produced and used for infection experiments as detailed previously (Meijer et al. 2006). Cell colonies resistant to anti-estrogens (1 μM 4-hydroxytamoxifen (OH-TAM, Sigma–Aldrich), 1 μM raloxifene (raloxifen hydrochloride, Sigma–Aldrich), or 100 nM ICI 182 780 (Zeneca Pharmaceuticals, Macclesfield, UK)) were picked and expanded in standard medium supplemented with 10% conditioned medium of CRIP cells (mouse fibroblast cell line) and the respective anti-estrogen (Dorssers et al. 1993). Soft agar colony assays in the presence of OH-TAM were performed as described (Meijer et al. 2006).

**Analysis of proviral cDNA inserts**

Integrated cDNAs were retrieved from genomic DNA by PCR using primers located adjacent to the cDNA cloning site and sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) using ABI PRISM BigDye Terminators v3.0 Cycle Sequencing Kits (Applied Biosystems) according to the protocols of the manufacturers as described (Meijer et al. 2006). The cDNAs were identified by sequence similarity searches.
Expression constructs and proliferation assays

cDNA clones of HRAS and RAF1 were recovered from the resistant cell lines and sequenced. The coding regions of HRAS (corresponding to nt 11 to 1029 of NM_005343.2), RAF1 (nt 883 to 2489 of NM_002880), PDGFRA (NM_006206), and PDGFRB (NM_002609.1) were cloned into the LZRS-IRE-Neo expression vector and introduced into ZR-75-1 cells as described (Meijer et al. 2006, Van Agthoven et al. 2009b). Two independently generated pools of cells were assayed for anti-estrogen resistance in 96-well plates using the WST-1 reagent (Roche Diagnostics) according to the recommendation of the supplier.

Patients

The protocol to study biological markers associated with disease outcome was approved by the medical ethics committee of the Erasmus Medical Center Rotterdam, The Netherlands (MEC 02.953). This retrospective study used 691 blind coded, ER protein-positive (≥ 10 fmol/mg protein) primary tumor tissues, in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands (http://www.fmvv.nl) and, wherever possible, has been reported in line with the REMARK guidelines (McShane et al. 2006). The primary breast tumors were from patients with detailed clinical followup as previously described (Van Agthoven et al. 2009a). All patients underwent breast surgery from 1979 to 1996. ER status was determined by routine ligand-binding assays or enzyme immunoassays (Foekens et al. 1989). For the study of tamoxifen resistance, ER-positive primary tumors of both lymph node-negative (LNN) and -positive patients were selected. Tumor aggressiveness was evaluated in LNN patients with ER-positive primary tumors who had not received any adjuvant systemic treatment. To evaluate the relationship of individual BCAR genes with tamoxifen resistance, 289 patients who received tamoxifen monotherapy as first-line treatment for advanced disease were included. Of these patients, 29 presented with distant metastasis at diagnosis. Of the remaining patients, none (41% were node negative) had received adjuvant hormone therapy, and 54 patients were treated with systemic adjuvant chemotherapy (20 with anthracycline and 34 with nonanthracycline-based regimens). Response to tamoxifen treatment was monitored according to standardized protocol (Hayward et al. 1977, EORTC Breast Cancer Cooperative Group 2000) as detailed previously (Van Agthoven et al. 2009a). Clinical benefit defined as objective response or stable disease (SD) for more than 6 months was achieved by 178 patients (61.6%) with 13 complete remissions, 38 partial remissions, and 127 SD for more than 6 months. No clinical benefit was observed for 111 patients (93 PD and 18 SD for 6 months or less). The median time to progression was 8.2 months. Median followup after start of therapy of patients alive at the end of the study (n = 70) was 43.1 months.

For the analysis of the association of individual BCAR genes with tumor aggressiveness, 518 LNN patients with ERα-positive tumors and who did not receive systemic adjuvant therapy were included. Fifty-three percent of the patients had undergone breast conserving lumpectomy and 100% node dissection. Adjuvant radiotherapy was given to 60% of the patients. Patients were evaluated every 3 months for the first 2 years, every 6 months for the next 3 years, and once a year thereafter. Distant metastases were recorded for 195 patients. The median followup of patients alive (n = 346) was 96.6 months and the median time to metastasis was 32 months. A total of 172 deaths were recorded.

Tissue processing

Primary tumor tissue processing was done as described previously (Sieuwerts et al. 2005). In summary, 20–60 cryostat sections of 30 μm, corresponding to 30–100 mg, were cut from frozen tissues for RNA isolation. To assess the amount of tumor cells relative to the amount of surrounding stromal cells, 5 μm sections were cut for hematoxylin and eosin staining, before, in between, and after cutting the sections for RNA isolation. For this study, only specimen with at least 30% tumor nuclei, distributed uniformly over at least 70% of the section area, was included.

Quantitative RT-PCR

RNA isolation, cDNA synthesis, and quantification of mRNA and quality control checks were done as described in detail before (Sieuwerts et al. 2005). RNA samples without distinct rRNA bands or failing to amplify efficiently with the housekeeper primer sets (see below) were excluded from this study. Real-time quantitative RT-PCR (qRT-PCR) was performed using an ABI Prism 7700 Sequence detection system (Applied Biosystems) and a Stratagene Mx3000P QPCR System (Agilent Technologies, Waldbronn, Germany). The prominent novel BCAR genes were selected for the development of primer sets designed to
detect the most abundant splice variants, quality controls, and pilot analyses in a small group of tumor specimens. Specific gene primer sets not meeting the stringent quality criteria (i.e., detection of genomic DNA or poor amplification efficiency) were excluded (Sieuwerts et al. 2005). The primer sets were used in combination with SYBR green PCR Master Mix (Applied Biosystems) and are described in Supplementary Table S1, see section on supplementary data given at the end of this article. Some gene quantifications were performed using inventoried TaqMan Gene Expression Assays from Applied Biosystems in combination with TaqMan Universal PCR Master Mix (Applied Biosystems) and in accordance with the protocol recommended by the manufacturer (Supplementary Table S1). ESR1 and PGR mRNA transcripts were measured as described before (Sieuwerts et al. 2005, 2007). To enable comparison of the mRNA levels in different samples, values were normalized using the average expression levels of a set of housekeeping genes containing HMBS, HPRT1, and B2M. Levels of the target genes expressed relative to this housekeeping set were quantified as follows: mRNA target \( 2^{(\text{mean } C_t \text{ housekeeping} - \text{mean } C_t \text{ target})} \) (Sieuwerts et al. 2005). \( C_t \) is defined as the detection threshold cycle number.

Statistical analyses

Statistical computations were done with the use of STATA statistical package, release 10.0 (STATA Corp., College Station, TX, USA). Differences in mRNA levels were assessed with the Mann–Whitney U test or Kruskal–Wallis test, including a Wilcoxon-type test for trend, when appropriate. In these tests, patient and tumor characteristics were used as grouping variables. The strengths of the associations between continuous variables were tested with the Spearman rank correlation \( (r_s) \). To reduce the skewness, variables were log transformed. All transformed data were normally distributed and analyzed as continuous variables or in quartiles. The Cox proportional hazard model was used to calculate the hazard ratio (HR) and 95% confidence interval (CI) in the analyses of distant metastasis-free survival (MFS), overall survival (OS), and progression-free survival (PFS). The proportional hazards assumptions were not violated as verified by using Schoenfeld residuals. MFS was defined as the time between removal of the primary tumor and the first detection of a distant metastasis, revealed after symptoms reported by the patients, occurrence of clinical signs, or at regular followup. Death from any cause was considered an event for OS. For all advanced patients treated with tamoxifen, PFS was defined as the time elapsed between initiation of tamoxifen and the first detection of progression of the disease. Logistic regression analysis was used to examine the relationship of mRNA levels with clinical benefit of tamoxifen therapy and for the calculation of the odds ratio (OR) and its 95% CI. A two-sided \( P \) value of \(<0.05\) was considered statistically significant.

Results

Functional screen identifies novel BCAR genes

We have previously reported the use of a rapid functional screen based on retrovirus-mediated transduction of expression cDNA libraries for the discovery of BCAR genes causing anti-estrogen-resistant cell proliferation (Meijer et al. 2006). These genes were identified following introduction of human placenta, brain, or mouse embryo cDNA libraries into ZR-75-1 cells and selection with 4-hydroxytamoxifen (OH-TAM, summarized in Tables 1 and 2).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>cDNA library</th>
<th>Anti-estrogen</th>
<th>Number of target cells (million)</th>
<th>Number of cell clones analyzed (number of different genes identified)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7\textsuperscript{a}</td>
<td>Brain</td>
<td>ICI 182 780</td>
<td>18 (agar)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>MCF-7\textsuperscript{a}</td>
<td>Brain/HELA</td>
<td>ICI 182 780</td>
<td>50</td>
<td>5 (3)</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Brain</td>
<td>ICI 182 780</td>
<td>50</td>
<td>23 (9)</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Brain\textsuperscript{b}</td>
<td>OH-TAM</td>
<td>30</td>
<td>45 (31)</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Brain/HELA</td>
<td>OH-TAM</td>
<td>18 (agar)</td>
<td>29 (19)</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Brain</td>
<td>Raloxifene</td>
<td>5</td>
<td>3 (2)</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>HELA</td>
<td>ICI 182 780</td>
<td>45</td>
<td>54 (44)</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Mouse embryo\textsuperscript{b}</td>
<td>OH-TAM</td>
<td>30</td>
<td>30 (11)</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>Placenta\textsuperscript{b}</td>
<td>OH-TAM</td>
<td>12</td>
<td>80 (31)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Approximately, 25% of the proliferating colonies survived transfer to microtiter plates and could be analyzed.

\textsuperscript{b}Details previously published (Meijer et al. 2006).
We extended these experiments by including a HELA cell line-derived cDNA library, another target cell line, and other culturing conditions including additional anti-estrogen agents. Based on the success of this screening protocol in ZR-75-1 cells (Meijer et al. 2006), infection experiments were carried out using the renowned MCF-7 cells as well. In pilot experiments using MCF-7 as a target and ICI 182 780 as anti-estrogen (Van Agthoven et al. 1998), only a few resistant cell clones were recovered (Table 1). The majority of these clones lacked an integrated retrovirus, indicating that spontaneous development of resistant colonies occurred at relatively high frequency in MCF-7 cells exposed to ICI 182 780. As a consequence, no further functional screens were performed using the MCF-7 cells.

In the experiments using the ZR-75-1 cell line as a target, all cell clones recovered from the different cultures were found to contain an integrated retrovirus, in agreement with the documented selectivity of this model (Dorssers et al. 1993, Van Agthoven et al. 2009b). From a total of 264 cell clones isolated from these selective cultures, 132 different inserted genes were identified (Table 1 and Supplementary Table S2, see section on supplementary data given at the end of this article). We previously defined a set of criteria for the identification of a BCAR gene causing the resistant phenotype. In brief, a specific gene has to represent the only detectable PCR product in at least one resistant cell clone and should be recovered from at least two cell clones arisen in independent infection events (Meijer et al. 2006). This is to ascertain that abundantly expressed genes, which are recovered from cell clones as passengers and are not responsible for the resistance, are not designated as BCAR genes. These criteria also preclude the possibility that insertion of a cDNA-containing virus leading to an insertion mutagenesis event, which may occur at a frequency of ~1 per 10 million infected cells (Van Agthoven et al. 2009b), is mistaken for a BCAR gene. Based on these criteria, our studies identified a total of 15 BCAR genes (ABCB1, BCAR4, CSFRI, EEF1AI, EGFR, EIF1, FBXL10, FGFI7, HRAS, NRG1, PDGFRA, PDGFRB, RAD21, RAF1, and RPL18A) individually responsible for anti-estrogen-resistant cell proliferation (Table 2). In most cases, a complete coding sequence lacking mutations was recovered for the respective genes, only the genes RAF1 and FBXL10 were consistently recovered as truncated cDNA fragments from the resistant cell clones (Table 2). In addition to these 15 BCAR genes, another 48 genes (Supplementary Table S2) did not meet these stringent criteria and are considered candidate BCAR genes, which require further studies to confirm their causative role in anti-estrogen-resistant cell proliferation. Already, one of these candidate genes (BCAR1) was previously identified by insertional mutagenesis and shown to cause tamoxifen resistance (Brinkman et al. 2000, Van der Flier et al. 2000).

### Recovery of individual BCAR genes depends on the biological selection

Initial experiments were performed using OH-TAM for selection of resistant colonies yielding different

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### Table 2: Representation of BCAR genes in anti-estrogen-resistant cell clones derived following transduction of ZR-75-1 cells with different cDNA libraries

<table>
<thead>
<tr>
<th>Selection</th>
<th>Brain</th>
<th>HE LA</th>
<th>Placenta</th>
<th>Mouse embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH-TAM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>FGF17 (12/45)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NRG1 (8/45)</td>
<td>NRG1 (52/80)</td>
<td>Pdgfrb (17/30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PDGFRB (12/80)</td>
<td>Pdgfra (7/30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EGFR (4/80)</td>
<td>Nrg1 (4/30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CSF1R (2/80)</td>
<td></td>
</tr>
<tr>
<td>OH-TAM/agar</td>
<td>NRG1 (3/18)</td>
<td>FBXL10 (3/18)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>HRAS (6/11)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAD21 (2/18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICI 182 780</td>
<td>NRG1 (15/23)</td>
<td>FGF17 (3/23)</td>
<td>HRAS (19/54)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RAF1 (4/54)&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RAD21, ABCB1,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RPL18A, EIF1,</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>EEF1A1 (2/54)</td>
<td></td>
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</tbody>
</table>

<sup>a</sup>Additional genes identified are listed in the Supplementary Table S2.

<sup>b</sup>Details previously published (Meijer et al. 2006).

<sup>c</sup>Number of cell clones containing the gene/total number of cell clones.

<sup>d</sup>All cDNA fragments lacking the 5'-terminal part coding for the JmjC domain.

<sup>e</sup>All cDNA fragments lacking the 5'-terminal part coding for the RAS-binding domain.
BCAR genes (Table 2). Further screens were carried out in which anti-estrogens other than OH-TAM were used. While the FGF17 and NRG1 genes predominated in the OH-TAM selection of cells transduced with the brain cDNA library, the NRG1 gene was more often recovered from cell clones selected with ICI 182 780 or raloxifene (Table 2). Cell clones transduced with the brain cDNA library were also selected for growth in semi-solid agar medium supplemented with OH-TAM. In contrast to the fluid culture, the FGF17 gene was recovered only once in these semi-solid cultures. In addition to three clones containing NRG1, two novel BCAR genes were identified (RAD21 and FBXL10). In experiments using the HELA cDNA library, HRAS predominated both in cell clones obtained from the selection using ICI 182 780 and semi-solid cultures. All together, these results show that by using various cDNA libraries, culture conditions, and anti-estrogens, different BCAR genes can be elucidated.

**Overexpression of HRAS, RAF1, and both PDGFRs induces anti-estrogen-resistant growth**

The HRAS gene was recovered from multiple cell clones following transduction of the HELA cDNA library (Table 2) and sequence analysis confirmed the absence of mutations in the integrated cDNAs. For further study, an expression construct was generated in the LZRS-IRES-Neo expression vector. ZR-75-1 cells transfected with the HRAS-expression construct showed efficient cell proliferation in the presence of OH-TAM, ICI 182 780, as well as raloxifene (Fig. 1A). Empty vector control cells failed to proliferate under these conditions. We have also transduced expression constructs containing the truncated form of RAF1, lacking the NH2-terminal RAS-binding site, and the complete coding regions from both PDGFR genes into ZR-75-1 cells. As shown in Fig. 1B, expression constructs of RAF1, PDGFRα, and PDGFRβ induced cell proliferation in the presence of the anti-estrogen OH-TAM, while vector control cells were fully growth inhibited. We also observed proliferation for these transduced cells in the presence of raloxifene and ICI 182 780 (data not shown). These results support the role of the HRAS, RAF1, PDGFRα, and PDGFRβ genes in estrogen-independent and anti-estrogen-resistant proliferation of ZR-75-1 cells.

**Evaluation of clinical relevance of novel BCAR genes**

Specimens of patients with ERα-positive disease were included for the quantification of BCAR mRNAs. In total, 289 tumors were analyzed from patients who received tamoxifen monotherapy as first-line treatment for advanced disease. In addition, tumor specimens of 518 LNN patients who had not received adjuvant systemic therapy were also available for the study of genes of interest. The mRNA levels of the novel BCAR genes EIF1, FBXL10, HRAS, NRG1, PDGFRα and PDGFRβ, RAD21, and RAF1 were determined with quantitative RT-PCR and normalized to a set of three housekeeping genes. Spearman’s rank correlation analyses revealed moderate to strong associations ($r_s > 0.40; P < 0.0001$) in mRNA levels between EIF1 and RAD21, between PDGFRα and PDGFRβ and NRG1, and between RAF1 and FBXL10 in these primary ER-positive tumors (Supplementary Table S3, see section on supplementary data given at the end of this article).
Associations of these biological factors with clinicopathological factors showed that reduced PDGFRA and NRG1 mRNA levels were associated with older age and post-menopausal status, and that low RAF1 mRNA levels were associated with poor grade (Supplementary Table S4, see section on supplementary data given at the end of this article).

Association of BCAR genes with tamoxifen resistance

For the evaluation of clinical benefit of first-line tamoxifen treatment, logistic regression analysis was performed on the continuous transformed mRNA levels or following division into four equal parts. Analysis as continuous variables showed high EIF1 mRNA levels to be associated with clinical benefit (OR = 2.59, \( P = 0.0007 \), Table 3). Similar results were obtained by analyzing the mRNA levels by quartiles showing the worst outcome for those patients with primary tumors categorized into the lowest quartile of EIF1 mRNA expression (Table 3). To evaluate the independent association of EIF1 mRNA expression with clinical benefit, the traditional predictive factors (age, menopausal status, disease-free interval, dominant site of relapse, and ESR1 and PGR mRNA levels)

### Table 3 Progression-free survival (PFS) and clinical benefit of 289 patients with advanced estrogen receptor-positive disease following first-line treatment with tamoxifen

<table>
<thead>
<tr>
<th></th>
<th>PFS</th>
<th>Clinical benefit</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td>Age at the start of therapy (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 40</td>
<td>0.0502</td>
<td>NS</td>
</tr>
<tr>
<td>41–55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>56–70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menopausal status at the start of therapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post</td>
<td></td>
<td></td>
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<tr>
<td>Disease-free interval (years)</td>
<td></td>
<td></td>
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<tr>
<td>≤ 1</td>
<td></td>
<td></td>
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<tr>
<td>1–3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dominant site of relapse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local regional</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td></td>
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<tr>
<td>Viscera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESR1 mRNA level</td>
<td>Continuous</td>
<td>0.0002</td>
</tr>
<tr>
<td>PGR mRNA level</td>
<td>Continuous</td>
<td>0.0049</td>
</tr>
<tr>
<td>Factors analyzed</td>
<td>Additions to the base model(a)</td>
<td>Additions to the base model(a)</td>
</tr>
<tr>
<td>EIF1 ((N = 226))</td>
<td>Continuous</td>
<td>0.0132</td>
</tr>
<tr>
<td>PDGFRA ((N = 283))</td>
<td>Continuous</td>
<td>0.0068</td>
</tr>
</tbody>
</table>

HR, hazard ratio; OR, odds ratio; CI, confidence interval; Q, quartile; NS = \( P > 0.1000 \).

\(a\)Factors were separately introduced to the base multivariate model that included the factors age, menopausal status, disease-free interval, dominant site of relapse, and ESR1 and PGR mRNA levels.

\(b\)For the analysis in quartiles, HR or OR was set at 1.00 for Q1.

\(c\)Stratified into high (Q2–Q4) versus low (Q1).
(Sieuwerts et al. 2007) were included in the analysis. In univariate and multivariate analyses, disease-free interval and ESR1 mRNA levels were significantly associated with clinical benefit. In a multivariate analysis including all the traditional predictive factors, EIF1 was an independent factor when analyzed as continuous variable or in quarters (Table 3). When combining the three highest quartiles in an exploratory analysis, high levels of EIF1 mRNA were associated with favorable outcome. While none of the other genes were significantly related when analyzed as continuous variable (Supplementary Table S5, see section on supplementary data given at the end of this article), categorized PDGFRA mRNA levels showed a significant association in univariate and multivariate analyses of clinical benefit (Table 3). Patients with primary tumors having mRNA levels in the third quartile (i.e. intermediate levels) showed the worst response on tamoxifen. Inclusion of adjuvant chemotherapy as an additional variable did not significantly change the estimates of the individual genes.

Association with PFS after the start of first-line treatment with tamoxifen was assessed with Cox regression analyses. When analyzed as continuous variable, low EIF1 mRNA levels were associated with an early progression (HR = 0.70, P = 0.013, Table 3). Similarly, patients stratified into four equal groups according to increasing mRNA levels showed a longer PFS (Table 3 and Supplementary Figure S1, see section on supplementary data given at the end of this article). An exploratory analysis revealed that patients with primary tumors containing the lowest quarter of EIF1 mRNA showed a median time to progression of 5.2 months, while the remaining patients with higher levels showed a median time to progression of 10 months after the start of the treatment (Fig. 2A). When combined with the traditional prognostic factors in a multivariate analysis, continuous and categorized EIF1 mRNA levels were significantly associated with PFS (Table 3). The mRNA levels of the other genes did not show statistically significant associations with PFS (Supplementary Table S5). In line with the findings for clinical benefit, categorized mRNA levels of PDGFRB revealed a nonlinear relationship with the largest risk for patients with PDGFRB mRNA levels in the third quartile. The Kaplan–Meier plot showed that half of these patients progressed within 5.6 months, while the remaining groups showed delayed progression (Fig. 2B). The observed associations of PDGFRB and EIF1 with PFS were independent of ER levels.

**Figure 2** Progression-free survival of ER-positive breast cancer patients with recurrent disease treated with first-line tamoxifen monotherapy. Kaplan–Meier curves for PFS for subgroups of patients as a function of the EIF1 (panel A) or PDGFRB (panel B) mRNA levels of the primary tumors. Patients were divided in groups having primary tumors with high (above the first quarter) or low EIF1 mRNA levels, or in four groups with low, moderate, intermediate, and high PDGFRB mRNA levels. Patients at risk at 24-month intervals are indicated. N, number of patients; F, number of patients showing progression.

**Association of BCAR genes with tumor aggressiveness**

We also determined whether PDGFRA and PDGFRB, HRAS, and RAF1 mRNA levels were associated with MFS and OS. For this analysis, we included 518 LNN patients with ERα-positive, primary tumors who had not received any adjuvant systemic treatment and thus reflect the natural course of the disease. Uni- and multivariate analyses showed that PDGFRA mRNA levels, analyzed as continuous or categorized variable, were significantly associated with MFS and OS, and
Table 4 Metastasis-free survival (MFS) and overall survival (OS) of 518 lymph node-negative patients with estrogen receptor-positive breast tumors

<table>
<thead>
<tr>
<th>Factors analyzed</th>
<th>MFS</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td></td>
<td>HR 95% CI</td>
<td>P</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤40</td>
<td>1</td>
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</tr>
<tr>
<td>41–55</td>
<td>0.78</td>
<td>0.51</td>
</tr>
<tr>
<td>56–70</td>
<td>0.58</td>
<td>0.37</td>
</tr>
<tr>
<td>&gt;70</td>
<td>0.46</td>
<td>0.28</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
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</tr>
<tr>
<td>Pre</td>
<td>1</td>
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</tr>
<tr>
<td>Post</td>
<td>0.71</td>
<td>0.54</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>≤2 cm</td>
<td>1.15</td>
<td>0.87</td>
</tr>
<tr>
<td>&gt;2 cm</td>
<td>1.11</td>
<td>0.82</td>
</tr>
<tr>
<td>Grade</td>
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</tr>
<tr>
<td>Poor</td>
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<td>1.00</td>
</tr>
<tr>
<td>Unknown</td>
<td>1.11</td>
<td>0.82</td>
</tr>
<tr>
<td>ESR1 mRNA</td>
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<tr>
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<td>0.89</td>
</tr>
<tr>
<td>PDGFRA mRNA</td>
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<td>0.84</td>
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<tr>
<td>Continuous</td>
<td>0.90</td>
<td>0.84</td>
</tr>
<tr>
<td>RAF1 mRNA</td>
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</tr>
<tr>
<td>Continuous</td>
<td>0.90</td>
<td>0.84</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval; Q, quartile; NS = P>0.1000.

aMFS was restricted to 60 months to avoid violation of the proportional hazards assumption.
bFactors were separately introduced to the base multivariate model that included the factors age, menopausal status, tumor size, grade, and ESR1 and PGR mRNA levels.
cFor the analysis in quartiles, HR was set at 1.00 for Q1.
dStratified into high (top 33%) and low.
eStratified into high (top 75%) and low.
independent of the traditional prognostic variables (age, menopausal status, tumor size, grade, and ESR1 and PGR mRNA levels; Table 4). The Kaplan–Meier analysis showed that patients with tumors containing the lowest levels (quartile 1) of PDGFRA mRNA had the best outcome (Supplementary Figure S2, see section on supplementary data given at the end of this article). When combining the patients with the higher levels of PDGFRA mRNA, their outcome was significantly worse compared with the group with low levels (Fig. 3A and Table 4). PDGFRB and RAF1 mRNA levels were not associated with MFS or OS (Table 4). HRAS, analyzed as a continuous variable in univariate analysis, was significantly associated with MFS in ER-positive tumors (Table 4). The analysis in quartiles revealed a nonsignificant shorter MFS for patients with tumors containing the highest levels of HRAS mRNA (Supplementary Figure S3, see section on supplementary data given at the end of this article).

An exploratory analysis, which compared patients with tumors having the highest levels of HRAS mRNA against the other patients, showed a worse prognosis for the group with the highest mRNA levels (Table 4 and Fig. 3B).

**Discussion**

Functional genetic screens are a powerful tool to identify specific genes contributing to a particular biological process. In our present study on the identification of genes underlying resistance to anti-estrogen compounds in breast cancer, we applied retroviral transduction of cDNA expression libraries, an effective and rapid technique (Brummelkamp & Bernards 2003, Meijer et al. 2006, Berns et al. 2007, Swanton et al. 2007, Whitehurst et al. 2007). We observed that the nature of the identified genes is strongly dependent on the tissue source and the quality of the cDNA library (Table 2). While the BCAR4 gene dominated in the transduction experiments performed with the placenta cDNA library, FGF17 and NRG1 were overrepresented in cell clones derived with the brain library, and HRAS was frequently recovered with the HELa cell library. These results show that use of different cDNA libraries is essential to get a wide representation of players in the studied phenotype. Obviously, it would be very attractive to use an expression library derived from tamoxifen-resistant breast tumors, but the stringent requirement for intact, full-length cDNAs is likely to present a major obstacle.

Our transfection experiments have shown that many BCAR genes provide resistance to tamoxifen as well as pure anti-estrogens (Van Agthoven et al. 2009b). However, in a screen based on colony formation using different culture conditions, the efficiency of individual genes may vary substantially. We observed that NRG1 was more frequently recovered from resistant colonies under selection with pure anti-estrogen (65%) than following selection with tamoxifen (27%), while the contribution of FGF17 was slightly reduced under the stringent conditions (from 18% with tamoxifen to 13% with ICI 182 780). These results suggest that growth signaling through the EGFR family members is more efficient than along the FGFR pathway under the more restrictive conditions. In the colonies obtained in semi-solid cultures, both NRG1 and FGF17 were relatively underrepresented and novel BCAR genes appeared...
(FBXL10 and RAD21). The probability of recovery of a particular gene under different selection conditions may depend on the integration position modulating its level of expression and on the consequences of the particular anti-estrogen on the biology of the ZR-75-1 cells. Our observations that the mechanisms underlying resistance against diverse anti-estrogens differ are in line with previous observations in breast cancer cell models (Soulez & Parker 2001, Faridi et al. 2003, Frasor et al. 2004, Martin et al. 2005, Fan et al. 2006, Kuske et al. 2006, Scafoglio et al. 2006, Shaw et al. 2006, Osipo et al. 2007). Accordingly, patients with breast tumors failing on tamoxifen have been shown to respond to the pure anti-estrogen fulvestrant (Howell et al. 2002, Osborne et al. 2002, Howell 2006), indicating subtle differences in the underlying mechanisms of tumor growth control. High throughput extension of these screens may help to elucidate the mechanistic differences between the diverse anti-estrogen agents used against breast cancer.

Our list of BCAR genes contains several genes (NRG1, FGF17, HRAS, RAF1, and the membrane receptors PDGFRA, PDGFRB, EGFR, and CSF1R), which are well documented with respect to their role in normal and malignant cell growth (Hunter 2000, Gschwind et al. 2004). In addition, RAS, RAF, and PDGFRA are frequently mutated in different types of cancer (Bos 1989, Emuss et al. 2005, Zebisch et al. 2006, Brugge et al. 2007). RAD21 is known to play a role in chromosome cohesion during cell cycle, homologous recombination-mediated double strand break repair, and apoptosis. Reduction of RAD21 expression inhibits proliferation and viability of breast cancer cells and increased their sensitivity to DNA-damaging agents (Atienza et al. 2005). The identification of RAD21 in the screen in ZR-75-1 cells suggests that its overexpression may accelerate the cell cycle and prevent anti-estrogen-induced apoptotic events. F-box proteins function as components of SCF-type E3 ubiquitin ligase complexes and may participate in co-repressor complexes (Gearhart et al. 2006). The presence of a histone demethylase activity in FBXL10 was shown to be important for regulation of cell senescence and transcription of rRNAs (Jin et al. 2004, Takeuchi et al. 2006, Frescas et al. 2007, He et al. 2008, Pfau et al. 2008, Yamagishi et al. 2008).

In our experiments, truncated versions of FBXL10 gene were recovered lacking the 5′-terminal part encoding the demethylase domain. As a consequence of its overexpression, this variant may overrule the function of the endogenous protein and enhance cell proliferation in anti-estrogen-treated breast cancer cells. How BCAR4 (Meijer et al. 2006) and the genes involved in mRNA translation (EIF1, EEF1A1, and RPL18A) induce anti-estrogen-resistant cell proliferation remains to be resolved.

The analyses for clinical relevance of several novel BCAR genes, revealed by retroviral transduction of cDNA expression libraries, showed significant associations with tamoxifen resistance for EIF1 and PDGFRA, and with tumor aggressiveness (i.e. metastatic potential) for HRAS and PDGFRA, although replication in an independent group of patients will be needed to obtain definite proof. EIF1 is a translation initiation factor involved in start codon selection (Fraser & Doudna 2007). While little was known about EIF1, other components of protein translation have been implicated in malignant processes involving either TP53 or acting downstream of PI3K/AKT/MTOR by selectively increasing translation of subsets of mRNAs (Armengol et al. 2007, MacInnes et al. 2008, Sonenberg 2008, Zhang et al. 2008, Chakraborty et al. 2009, Lai et al. 2009). Furthermore, EEF1A protein levels were increased in breast tumors with high proteasome activity (Chen & Madura 2005). Low EIF1 mRNA levels in breast tumors are associated with shorter PFS and poor clinical benefit of tamoxifen treatment, while high levels cause the resistance in the in vitro cell model. Similar observations were made for BCAR3 and TLE3 (Van Aghoven et al. 2009a), indicating that our cell model cannot fully replicate the clinical situation, known to be heterogeneous. Mutations in PDGFRA, yielding constitutive activation of this receptor, drive malignant behavior in gastrointestinal stromal tumors (Heinrich et al. 2003, Hirota et al. 2003). Both PDGFRs have been implicated in the invasive potential of breast tumors (Jechlinger et al. 2006, Paulsson et al. 2009). In breast cancer, our results showed an association of high PDGFRA mRNA levels with rapid recurrence of the disease. Furthermore, advanced breast cancer patients with primary tumors with mRNA levels in the third quartile did worse in terms of clinical benefit and PFS. These results may suggest that patients with ER-positive tumors containing PDGFRA in the third quartile are unlikely to benefit from adjuvant tamoxifen treatment. In contrast to PDGFRA mRNA levels, we did not observe any relationship between breast cancer progression and tamoxifen resistance for PDGFRB. HRAS is rarely mutated in breast cancer (Hollestelle et al. 2007), but high mRNA levels were found to be associated with increased metastatic potential in untreated patients in line with its reported role in progression and invasion (Watson et al. 1991, Gelmann et al. 1992, Moon et al. 2000, Worsham et al. 2006).
No associations of RAF1 or NRG1 mRNA levels with tamoxifen resistance were observed in our study. Recently, RAF1 has been shown to be a direct target for down-regulation by miR-7 (Webster et al. 2009), and high levels of this miRNA have been linked to poor prognosis of breast cancer patients (Foekens et al. 2004). Furthermore, RAF1 has been suggested to contribute to tamoxifen resistance induced by the loss of CDK10 in MCF-7 cells (Iorns et al. 2008). Although only incomplete versions of RAF1 were recovered from our current screen, it is uncertain whether the loss of the RAS-binding domain is a pre-requisite for anti-estrogen-resistant growth of the breast cancer cells. NRG1 was previously shown to induce tamoxifen-resistant and invasive growth (Atlas et al. 2003), and is expressed at variable levels and amplified in breast tumors (Huang et al. 2004, Prentice et al. 2005, Hutcheson et al. 2007). These published associations suggest that RAF1 or NRG1 mRNA levels are not decisive in clinical tamoxifen resistance and that post-transcriptional regulation may be more important for these BCAR genes. Alternatively, the functional partners of BCAR genes may be rate limiting and exhibit significant associations as previously noted for FGF17/FGFR4 (Meijer et al. 2008).

In conclusion, the recovery frequencies of individual BCAR genes upon selection with various anti-estrogens illustrate the biological differences of these drugs for cellular resistance to growth inhibition. Further large-scale transduction experiments may characterize these fundamental differences and provide rationale for the prescription of an appropriate drug to a given patient. Furthermore, the clinical associations of the different BCAR genes identified in our cell model (Van der Flier et al. 2000, Dorssers et al. 2004a,b, Meijer et al. 2008, 2009, Van Agthoven et al. 2009a,c) and their mode of action will provide insights into the mechanisms of tumor aggressiveness and endocrine therapy response, and may help to design improved treatments aiming at prolonged control of the disease.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-09-0062.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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