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

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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*, *PDGFRA*, *PDGFRB*, *RAD21*, and *RAF1*) with tamoxifen treatment in patients with advanced disease showed significant association with clinical benefit and progression-free survival for *EIF1* and *PDGFRA* mRNA levels. Furthermore, *PDGFRA* 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 ER α -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 thrombo-embolic 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). In spite of the considerable progress made in the last decades, we still do not comprehend the complete spectrum of resistance mechanisms, and detailed study of different models may help to resolve these options.

In recent years, we have used functional genetic screens to identify individual genes that contribute to or are responsible for resistance to anti-estrogens. Our first approach was based on insertional mutagenesis by targeting cells with defective retroviruses. The integration of virus can affect an individual gene and thereby altering the cell phenotype (Dorssers *et al.* 1993). Through this approach, seven breast cancer anti-estrogen resistance (*BCAR*) genes responsible for the transition of an estrogen-dependent, tamoxifen-sensitive human breast cancer cell line into a tamoxifen-resistant phenotype have been elucidated (Van Agthoven *et al.* 1998, 2009b, Brinkman *et al.* 2000). In view of the complexity and the workload of this approach, we have recently tested retroviral transduction of cDNA expression libraries (Brummelkamp & Bernards 2003) as an alternative strategy. In these experiments, the coding sequences of numerous genes present in the library are introduced into target cells by means of retroviral infection. Cells that acquired a gene product enabling growth in the presence of drugs can be recovered and the gene conferring resistance can be identified through PCR.

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 17 β -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 μ M 4-hydroxytamoxifen (OH-TAM, Sigma–Aldrich), 1 μ M raloxifene (raloxifene 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

using Basic Local Alignment Search Tools (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

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-IRES-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 ER α -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 μ m, 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 μ m 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: $\text{mRNA target} = 2^{(\text{mean } C_t \text{ housekeeping} - \text{mean } C_t \text{ target})}$ (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 1 Functional screens for anti-estrogen-resistant cell proliferation

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

^aApproximately, 25% of the proliferating colonies survived transfer to microtiter plates and could be analyzed.

^bDetails previously published (Meijer *et al.* 2006).

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

Selection	Brain	HELA	Placenta	Mouse embryo
OH-TAM ^b	<i>FGF17</i> (12/45) ^c <i>NRG1</i> (8/45)		<i>BCAR4</i> (52/80) <i>PDGFRB</i> (12/80) <i>EGFR</i> (4/80) <i>CSF1R</i> (2/80)	<i>Pdgfrb</i> (17/30) <i>Pdgfra</i> (7/30) <i>Nrg1</i> (4/30)
OH-TAM/agar	<i>NRG1</i> (3/18) <i>FBXL10</i> (3/18) ^d <i>RAD21</i> (2/18)	<i>HRAS</i> (6/11)		
ICI 182 780	<i>NRG1</i> (15/23) <i>FGF17</i> (3/23)	<i>HRAS</i> (19/54) <i>RAF1</i> (4/54) ^e <i>RAD21</i> , <i>ABCB1</i> , <i>RPL18A</i> , <i>EIF1</i> , <i>EEF1A1</i> (2/54)		
Raloxifene	<i>NRG1</i> (3/3)			

^aAdditional genes identified are listed in the Supplementary Table S2.^bDetails previously published (Meijer *et al.* 2006).^cNumber of cell clones containing the gene/total number of cell clones.^dAll cDNA fragments lacking the 5'-terminal part coding for the JmjC domain.^eAll cDNA fragments lacking the 5'-terminal part coding for the RAS-binding domain.

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 renown 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*, *CSF1R*, *EEF1A1*, *EGFR*, *EIF1*, *FBXL10*, *FGF17*, *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

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*, *PDGFRA*, and *PDGFRB* 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*, *PDGFRA*, and *PDGFRB* 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 ER α -positive disease were included for the quantification of *BCAR* mRNAs. In total, 289 tumors were analyzed from patients who received

Figure 1 Anti-estrogen resistance of ZR-75-1 cells with forced expression of *BCAR* genes. (A) Cells transfected with the *HRAS* expression vector (solid line) or the empty vector (LZRS, dashed line) were seeded at equal density (2500 cells/well) and cultured with OH-TAM (○), ICI 182 780 (▲), or raloxifene (X). Proliferation assays were performed at the indicated time points on eight wells for each condition. Mean absorbance and SD are presented. (B) Independent cell pools transduced with expression vectors containing cDNAs for *PDGFRA* (◇, ◆), *PDGFRB* (○, ●), *RAF1* (▲, △), or empty vectors (■, □) were cultured with OH-TAM and measured at the indicated time points as described above.

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*, *PDGFRA* and *PDGFRB*, *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 *PDGFRA* and *PDGFRB* 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 clinico-pathological 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

	PFS								Clinical benefit							
	Univariate				Multivariate				Univariate				Multivariate			
	HR	95% CI		P	HR	95% CI		P	OR	95% CI		P	OR	95% CI		P
Age at the start of therapy (years)				0.0502				NS					NS			NS
≤40	1				1				1				1			
41–55	0.77	0.46	1.29		0.67	0.39	1.15		0.83	0.29	2.37		0.71	0.23	2.22	
56–70	0.65	0.39	1.10		0.52	0.27	0.99		1.29	0.45	3.66		0.67	0.17	2.64	
>70	0.53	0.31	0.91		0.44	0.22	0.86		1.43	0.48	4.22		0.80	0.20	3.24	
Menopausal status at the start of therapy				NS				NS					0.0544			NS
Pre	1				1				1							
Post	0.85	0.64	1.12		1.30	0.86	1.97		1.70	0.99	2.90		1.48	0.63	3.52	
Disease-free interval (years)				0.0006				0.0004					<0.0001			<0.0001
≤1	1				1				1				1			
1–3	0.67	0.50	0.90		0.66	0.49	0.89		3.29	1.81	5.97		3.41	1.83	6.36	
>3	0.52	0.38	0.72		0.50	0.36	0.70		3.85	1.99	7.43		4.04	2.02	8.11	
Dominant site of relapse				NS				NS					NS			NS
Local regional	1				1				1				1			
Bone	1.25	0.84	1.85		1.15	0.76	1.75		0.59	0.26	1.33		0.57	0.24	1.37	
Viscera	1.15	0.76	1.73		1.26	0.82	1.94		0.79	0.34	1.83		0.64	0.26	1.59	
ESR1 mRNA level																
Continuous	0.90	0.85	0.95	0.0002	0.91	0.85	0.97	0.0004	1.21	1.08	1.35	0.0008	1.21	1.06	1.38	0.0053
PGR mRNA level																
Continuous	0.90	0.84	0.97	0.0049	0.92	0.85	0.99	0.0290	1.09	0.94	1.26	NS	1.02	0.86	1.20	NS
Factors analyzed					Additions to the base model ^a								Additions to the base model ^a			
EIF1 (N=226)																
Continuous	0.70	0.53	0.93	0.0132	0.80	0.60	1.06	NS	2.59	1.46	4.61	0.0007	2.61	1.35	5.05	0.0033
In quartiles ^b				0.0593				NS				0.0108				0.0285
Q2 versus Q1	0.63	0.43	0.93		0.62	0.42	0.93		2.28	1.08	4.85		2.13	0.93	4.89	
Q3 versus Q1	0.66	0.45	0.97		0.65	0.44	0.97		2.53	1.19	5.39		2.73	1.19	6.29	
Q4 versus Q1	0.62	0.42	0.91		0.72	0.48	1.07		3.39	1.56	7.38		3.37	1.40	8.12	
High versus low ^c	0.64	0.46	0.87	0.0068	0.66	0.48	0.92	0.0160	2.69	1.45	4.97	0.0015	2.66	1.34	5.28	0.0047
PDGFRA (N=283)																
Continuous	1.10	0.95	1.28	NS	1.00	0.86	1.17	NS	0.82	0.61	1.11	NS	0.97	0.69	1.35	NS
In quartiles ^b				0.0718				0.0232				0.0108				0.0017
Q2 versus Q1	1.00	0.71	1.41		1.06	0.74	1.52		0.76	0.38	1.53		0.76	0.35	1.63	
Q3 versus Q1	1.50	1.07	2.10		1.58	1.11	2.24		0.35	0.18	0.70		0.34	0.16	0.72	
Q4 versus Q1	1.19	0.85	1.67		0.93	0.64	1.33		0.88	0.44	1.79		1.40	0.64	3.10	

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

^aFactors 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.

^bFor the analysis in quartiles, HR or OR was set at 1.00 for Q1.

^cStratified 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 *PDGFRA* revealed a nonlinear relationship with the largest risk for patients with *PDGFRA* 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 *PDGFRA* 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 *PDGFRA* (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 *PDGFRA* 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 ER α -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

	MFS ^a						OS					
	Univariate			Multivariate			Univariate			Multivariate		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age (years)			0.0085			0.089			NS			NS
≤40	1			1			1			1		
41–55	0.78	0.51 1.20		0.81	0.52 1.26		0.90	0.55 1.47		0.93	0.56 1.55	
56–70	0.58	0.37 0.91		0.48	0.25 0.95		0.84	0.50 1.39		0.65	0.31 1.38	
>70	0.46	0.28 0.78		0.38	0.18 0.80		1.12	0.66 1.91		0.93	0.42 2.04	
Menopausal status			0.0196			NS			NS			NS
Pre	1			1			1			1		
Post	0.71	0.54 0.95		1.11	0.65 1.90		1.10	0.81 1.49		1.22	0.67 2.23	
Tumor size			NS			NS			NS			NS
≤2 cm	1			1			1			1		
>2 cm	1.15	0.87 1.53		1.16	0.87 1.55		1.22	0.90 1.65		1.14	0.83 1.55	
Grade			0.0035			0.0019			NS			NS
Poor	1			1			1			1		
Unknown	1.11	0.82 1.51		1.26	0.91 1.73		1.00	0.72 1.40		1.06	0.74 1.50	
Moderate/good	0.55	0.36 0.84		0.58	0.37 0.90		0.65	0.42 1.00		0.71	0.46 1.10	
<i>ESR1</i> mRNA												
Continuous	0.95	0.89 1.00	0.0652	1.03	0.96 1.11	NS	0.98	0.92 1.04	NS	1.02	0.95 1.10	NS
<i>PGR</i> mRNA												
Continuous	0.90	0.84 0.96	0.0014	0.90	0.83 0.96	0.0028	0.89	0.83 0.95	0.0008	0.88	0.81 0.95	0.0002
Factors analyzed				Additions to the base model^b						Additions to the base model^b		
<i>HRAS</i> (N=462)												
Continuous	1.33	1.01 1.76	0.0429	1.25	0.95 1.65	NS	1.23	0.95 1.61	NS	1.15	0.88 1.50	NS
In quartiles ^c			NS			NS			NS			NS
Q2 versus Q1	0.97	0.60 1.58		0.97	0.59 1.59		1.00	0.64 1.58		0.92	0.58 1.46	
Q3 versus Q1	0.99	0.61 1.60		0.92	0.57 1.50		0.97	0.61 1.53		0.87	0.54 1.38	
Q4 versus Q1	1.45	0.93 2.25		1.32	0.84 2.08		1.22	0.80 1.88		1.10	0.71 1.70	
High versus low ^d	1.66	1.16 2.23	0.0052	1.49	1.06 2.07	0.0220	1.36	0.99 1.86	0.0598	1.28	0.94 1.77	NS
<i>PDGFRA</i> (N=512)												
Continuous	1.28	1.07 1.54	0.0072	1.32	1.09 1.60	0.0047	1.15	0.97 1.37	NS	1.24	1.04 1.47	0.0175
In quartiles ^c			0.0040			0.0016			NS			0.0080
Q2 versus Q1	1.89	1.14 3.13		2.12	1.26 3.57		1.47	0.93 2.31		1.81	1.14 2.88	
Q3 versus Q1	2.15	1.31 3.52		2.25	1.35 3.73		1.53	0.97 2.41		1.83	1.15 2.92	
Q4 versus Q1	2.20	1.34 3.60		2.48	1.48 4.15		1.65	1.06 2.57		2.12	1.33 3.38	
High versus Low ^e	2.08	1.34 3.21	0.0004	2.27	1.45 3.57	0.0001	1.55	1.05 2.27	0.0203	1.90	1.28 2.84	0.0009
<i>PDGFRB</i> (N=490)												
Continuous	1.00	0.83 1.20	NS	1.09	0.90 1.31	NS	1.02	0.86 1.22	NS	1.07	0.90 1.28	NS
In quartiles ^c			NS			NS			NS			NS
Q2 versus Q1	0.94	0.60 1.48		1.20	0.75 1.91		1.08	0.70 1.67		1.17	0.75 1.83	
Q3 versus Q1	0.94	0.60 1.47		1.10	0.69 1.75		1.07	0.69 1.67		1.14	0.73 1.79	
Q4 versus Q1	1.02	0.66 1.60		1.32	0.82 2.10		1.14	0.73 1.77		1.31	0.83 2.07	
<i>RAF1</i> (N=461)												
Continuous	1.15	0.80 1.67	NS	1.17	0.80 1.72	NS	1.16	0.82 1.65	NS	1.15	0.80 1.65	NS
In quartiles ^c			NS			NS			NS			NS
Q2 versus Q1	1.02	0.64 1.62		1.12	0.70 1.81		0.87	0.55 1.37		1.00	0.63 1.60	
Q3 versus Q1	1.03	0.65 1.65		1.02	0.63 1.64		1.25	0.82 1.92		1.23	0.80 1.91	
Q4 versus Q1	1.07	0.68 1.69		1.08	0.67 1.76		0.96	0.62 1.50		0.99	0.62 1.58	

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

Figure 3 Metastasis-free survival of lymph node-negative patients with ER-positive breast cancer. Kaplan–Meier curves for MFS for subgroups of patients as a function of the *PDGFRA* (panel A) or *HRAS* (panel B) mRNA levels of the primary tumors. Patients were divided in two groups having primary tumors with high (*PDGFRA* top 75%, *HRAS* top 33%) or low mRNA levels. Patients at risk at 24-month intervals are indicated. N, number of patients; F, number of patients with distant recurrences.

(*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, Frasar *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 Aghthoven *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.* 2008). 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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