Functional Screen for Genes Responsible for Tamoxifen Resistance in Human Breast Cancer Cells

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Abstract

Antiestrogens, such as tamoxifen, are widely used for endocrine treatment of estrogen receptor–positive breast cancer. However, as breast cancer progresses, development of tamoxifen resistance is inevitable. The mechanisms underlying this resistance are not well understood. To identify genes involved in tamoxifen resistance, we have developed a rapid screening method. To alter the tamoxifen-sensitive phenotype of human ZR-75-1 breast cancer cells into a tamoxifen-resistant phenotype, the cells were infected with retroviral cDNA libraries derived from human placenta, human brain, and mouse embryo. Subsequently, the cells were selected for proliferation in the presence of 4-hydroxy-tamoxifen (OH-TAM) and integrated cDNAs were identified by sequence similarity searches. From 155 OH-TAM-resistant cell colonies, a total of 25 candidate genes were isolated. Seven of these genes were identified in multiple cell colonies and thus cause antiestrogen resistance. The epidermal growth factor receptor, platelet-derived growth factor receptor-α, platelet-derived growth factor receptor-β, colony-stimulating factor 1 receptor, neuregulin1, and fibroblast growth factor 17 that we have identified have been described as key regulators in the mitogen-activated protein kinase pathway. Therefore, this pathway could be a valuable target in the treatment of patients with breast cancer resistant to endocrine treatment. In addition, the putative gene *LOC400500*, predicted by *in silico* analysis, was identified. We showed that ectopic expression of this gene, designated as breast cancer antiestrogen resistance 4 (*BCAR4*), caused OH-TAM resistance and anchorage-independent cell growth in ZR-75-1 cells and that the intact open reading frame was required for its function. We conclude that retroviral transfer of cDNA libraries into human breast cancer cells is an efficient method for identifying genes involved in tamoxifen resistance.
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Introduction

Tamoxifen is the most extensively used antiestrogen in the treatment of breast cancer. Patients with estrogen receptor (ER)-positive breast tumors may initially benefit from this treatment, but almost all responding patients acquire resistance to the action of tamoxifen over time and the disease progresses. Several mechanisms for this phenomenon have been suggested, including alteration of the availability or metabolism of tamoxifen, alterations in the function of the ER and in the ER signaling cascade, and the altered expression of different genes (reviewed by refs. 1, 2). However, in the majority of patients, the mechanisms causing tamoxifen-resistant proliferation remain unexplained. Insight into these processes is essential for the development of improved treatment strategies and may be obtained by the application of genome-wide functional screens.

Random transfection of cDNA libraries was previously used to identify the specific genes involved in progression to antiestrogen resistance of human breast cancer cells, but this had only limited success (3). On the other hand, our group has successfully identified such genes using retroviral insertion mutagenesis in functional screens (4). Although insertion mutagenesis has shown itself to be a very powerful tool in the identification of genes involved in mouse tumorigenesis, in our experiments the identification of the genes responsible for antiestrogen-resistant proliferation has proved to be very labor intensive (5-7).

To bypass this technical limitation, we have developed a rapid screening strategy for identifying genes that cause tamoxifen-resistant cell proliferation. In this study, we used replication-defective retroviruses to express cDNAs, a strategy that has previously been used for various experimental purposes (8-10). We applied this methodology to estrogen-dependent human ZR-75-1 breast cancer cells and here report the identification of several genes that may be key players in the resistance of human breast cancer cells to antiestrogenic drugs.

Results

Functional Screens for Tamoxifen Resistance

ZR-75-1 human breast cancer cells were infected with retroviral cDNA libraries derived from either human placenta, human brain, or mouse embryo (Fig. 1). After infection, the

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cDNA library	no of cells	no of colonies	no of cDNAs
human placenta	1.2×10^7	80	31
mouse embryo	3×10^7	30	11
human brain	3.2×10^7	45	31
total	7.4×10^7	155	73

Criterion 1: single PCR product

25 candidate genes (Table 1)

Criterion 2: two independent cell colonies

7 genes causing tamoxifen resistance

FIGURE 1. Study outline. Functional screen for tamoxifen resistance. ZR-75-1 cells were transduced with retroviral cDNA libraries derived from human placenta (three independently infected cell cultures), mouse embryo (three independently infected cell cultures), and human brain (32 independently infected cell cultures). A total of 73 different genes were identified. In 25 colonies, only one integrated cDNA was observed, suggesting a causative role in tamoxifen resistance for the genes identified (Table 1). Seven of these genes were isolated from at least two independently derived cell colonies, complying with our second criterion.

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cells were selected for growth in the presence of 4-hydroxytamoxifen (OH-TAM). The first resistant colonies were observed 3 weeks after retroviral transduction, and all colonies were picked within 6 weeks after start of the experiments. Three independent cell cultures (a total of 1.2×10^7 cells) were infected with the human placenta cDNA library, and 80 tamoxifen-resistant cell colonies were isolated. In addition, 30 cell colonies were isolated from three flasks with independent cell cultures (10^7 cells each) infected with the mouse embryo cDNA library. To prevent the isolation of many identical cell colonies due to reseeding of proliferating cells, 32 individual 25 cm^2 flasks with $\sim 10^6$ cells each (a total of 3.2×10^7 cells) were infected with viral particles derived from a human brain library. In total, 45 cell colonies were isolated from the cell cultures infected with this library. In control experiments, ZR-75-1 cells mock infected or infected with pLIB-EGFP virus failed to produce proliferating colonies in the presence of OH-TAM within 6 weeks. In total, 7.4×10^7 cells were transduced with viral particles of three different cDNA libraries, resulting in 155 resistant cell colonies. The estimated infection frequency was $\sim 10\%$; thus, the frequency of cell colonies was at least 2,000-fold higher than that of spontaneous antiestrogen-resistant cell colonies in control cultures (below 10^{-8}; ref. 4).

Genes Recovered from Screens for Tamoxifen Resistance

Genomic DNA was isolated from resistant cell colonies, and integrated cDNAs were recovered using PCR assays. These assays showed that the majority of the tamoxifen-resistant cell colonies contained only one retroviral insert (range, 1-6; median, 1). Approximately one third of the isolated colonies contained multiple retroviral integrations.

In one of the OH-TAM-resistant cell lines obtained after infection with the placenta cDNA library, PCR analysis failed to detect an integrated cDNA. Southern blot analysis showed that this particular cell line contained an integrated retrovirus with only one retroviral long terminal repeat, likely the result of a truncation event during integration of the retrovirus in the host genome. The integrated cDNA in this cell line was successfully isolated using an inverse DNA amplification technique (11, 12). This phenomenon indicates that not every integrated cDNA may be detected by PCR analysis using primers located adjacent to the inserted cDNAs. Therefore, truncated retroviruses may lead to misconceived conclusions for some of the genes identified in a screen. In addition, we have shown previously that tamoxifen-resistant cell colonies may result from insertion mutagenesis of a retrovirus independent of its cDNA insert at a frequency of one colony per ~ 2 million cells (4). To ascertain that an inserted cDNA was actually responsible for tamoxifen resistance and to exclude wrong assignments as a consequence of virus truncation or insertion mutagenesis events, we applied the following criteria. First, each candidate cDNA represented a single product following 35 cycles of PCR with genomic DNA from at least one tamoxifen-resistant cell colony. Second, a particular cDNA should be recovered from at least two independently derived cell colonies. This independence was established by differences in length of the 5′-noncoding region of the inserted cDNAs or if cell colonies originated from infections of independent cell cultures. Analyses of the cDNAs isolated from the resistant colonies revealed a total of 73 different genes (Fig. 1). Twenty-five genes (listed in Table 1) complied with the first criterion and are considered candidate genes. Seven of these genes also complied with the second criterion and thus caused tamoxifen resistance in ZR-75-1 cells.

We fully sequenced the cDNA inserts of two independent colonies of each of these seven genes and confirmed the presence of cDNAs containing the complete open reading frame lacking mutations.

Among the seven genes causing tamoxifen resistance, four different receptor tyrosine kinases (RTK) were present: epidermal growth factor receptor (*EGFR*), colony-stimulating factor 1 receptor (*CSF1R*), mouse platelet-derived growth factor (PDGF) receptor-α (Pdgfra), and both mouse and human PDGF receptor-β (*Pdgfrb* and *PDGFRB*). Four cell colonies with integrated EGFR cDNAs were isolated from one cell culture infected with the human placenta library, as were two *CSF1R* cDNAs. Length differences at the 5′-end detected by sequence analysis confirmed their independent origin. Six of seven *Pdgfra* cDNAs were recovered from two independent cell cultures infected with the mouse embryo library. *PDGFRB* was identified in 12 colonies retrieved from one cell culture infected with the placenta library. At least two cell colonies had an independent origin because the inserted cDNAs differed at the 5′-end. Seventeen *Pdgfrb* cDNAs were isolated from resistant colonies retrieved from cell cultures infected with the mouse embryo library. Fourteen of these differed at the 5′-end or were recovered from autonomous cell cultures and have thus arisen independently. In addition to the RTK genes, we identified mouse and human neuregulin1 (*Nrg1* and *NRG1*) and human fibroblast growth factor (*FGF*) 17. The *Nrg1* gene was isolated four times from two independent cell cultures infected with the mouse embryo library, and the human *NRG1* was isolated eight times from six cell cultures independently infected with the brain library. All cell colonies contained the *NRG1* sensory and motor neuron-derived factor isoform SMDF. *FGF17* was identified in 12 colonies isolated from eight independent cell cultures infected with the human brain library.

LOC400500 [assigned as breast cancer antiestrogen resistance 4 (*BCAR4*), see Discussion], a putative gene predicted by *in silico* analysis of the human genome (Hs.24611, accession no. XM_378564), was the cDNA most frequently found. Based on sequence and Southern blotting analysis of PCR products, we recovered *LOC400500* 52 times only in the different cell cultures infected with the human placenta library. At least 26 of these cDNAs were proven to be of independent origin. Sequence similarity analyses showed that the cDNAs had variable 5′-ends. However, all cDNAs contained an open reading frame encoding a 121-amino acid polypeptide identical to the open reading frame predicted for *LOC400500* gene.

Ectopic Expression of LOC400500/BCAR4 *Induces Antiestrogen Resistance and Anchorage-Independent Transformation*

Additional experiments were done to confirm that the putative gene *LOC400500* is able to induce tamoxifen-resistant proliferation in human ZR-75-1 breast cancer cells. ZR-75-1 cells were

Table 1. Summary of the Genes Identified in the Screen

Gene	National Center for Biotechnology Information UniGene	No. total isolates*	No. independent isolates †	Library	Function ‡
LOC400500	Hs.24611	52	26	Human placenta	Unknown
Pdgfrb	Mm.4146	17	14	Mouse embryo	RTK
PDGFRB	Hs.509067	12	2	Human placenta	RTK
FGF17	Hs.248192	12	8	Human brain	Ligand of RTK
NRG1 §	Hs.453951	8	6	Human brain	Ligand of RTK
Nrg1 §	Mm.153432	4	2	Mouse embryo	Ligand of RTK
Pdgfra	Mm.221403	7	6	Mouse embryo	RTK
EGFR	Hs.488293	4	2	Human placenta	RTK
CSF1R	Hs.483829	2	2	Human placenta	RTK
Psap	Mm.277498	2	1	Mouse embryo	Enzyme activator
ASMTL	Hs.533514	2	1	Human placenta	Unknown
ALK	Hs.196534	2	1	Human brain	RTK
APLP1	Hs.74565	1	1	Human brain	Protein binding
APP	Hs.434980	1	1	Human brain	Signaling
CBFA2T3	Hs.513811	1	1	Human brain	Transcription
ERBB2	Hs.446352	1	1	Human placenta	RTK
GFAP	Hs.514227	1	1	Human brain	Intermediate filament
GFI1B	Hs.118539	1	1	Human placenta	Zinc finger protein
NEDD9	Hs.37982	1	1	Human placenta	Signaling
HRAS	Hs.37003	1	1	Human placenta	Signaling
KIAA0513	Hs.301658	1	1	Human brain	Unknown
L1CAM	Hs.522818	1	1	Human brain	Cell adhesion
CLDN23	Hs.183617	1	1	Human placenta	Cell adhesion
MDC1	Hs.433653	1	1	Human brain	DNA repair
PB1	Hs.189920	1	1	Human brain	DNA binding
PTMA	Hs.459927	1	1	Human placenta	Transcription
STX1A	Hs.488683	1	1	Human brain	Protein binding

NOTE: Inserted cDNAs were listed when they were the only product after 35 PCR cycles in the analysis of at least one cell colony. Seven of these cDNAs were found at least twice as independent event.
*Number of cell colonies identified with the respective gene.
†Number of independent cDNAs found using a particular cDNA library.
‡Function was derived from the National Center for Biotechnology Information/Gene database.
§Only transcript variant SMDF was identified.

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infected with retroviruses containing either an LZRS-IRES-Neo/ LOC400500 expression construct or the LZRS-IRES-Neo expression vector without insert, designated as ZR/BCAR4 and ZR/LZRS, respectively. After infection, cells were selected with G418 in 17β-estradiol (E_2)–containing medium. Proliferation of the different cell pools, originating from $\sim 10^5$ colonies each, was similar in the presence of E_2-supplemented medium (Fig. 2A). We next determined the capacity of the transduced cells to proliferate in the presence of OH-TAM. In Fig. 2B, proliferation curves over a 21-day culture period of these cells are presented. These curves show the dominant role of *LOC400500/ BCAR4* in tamoxifen-resistant proliferation of ZR-75-1 cells.

To establish whether the protein predicted for *LOC400500/ BCAR4* is responsible for tamoxifen resistance, a frameshift mutation was introduced using site-directed mutagenesis. This resulted in the addition of 20 heterologous amino acids after amino acid position 5 before reaching a stop codon. ZR-75-1 cell cultures were infected in duplicate with virus containing this frameshift construct (ZR/BCAR4-fs). Parallel infections were done in duplicate with BCAR4 or LZRS virus to generate control cultures (ZR/BCAR4 and ZR/LZRS, respectively). Two days after infection, 5% of the cells were plated in medium containing OH-TAM. In the ZR/BCAR4 cultures, proliferating cell colonies (average of 4,100 per flask) were observed within 3 weeks, whereas no proliferating cell colonies could be detected in ZR/BCAR4-fs cultures and in ZR/LZRS control cultures up to 5 weeks. Another 5% of the cells were plated in medium containing E_2 plus G418, and successful infection was shown by the presence of G418-resistant colonies in ZR/ BCAR4, ZR/BCAR4-fs, and ZR/LZRS cell cultures (average of 5,000, 3,950, and 4,150 colonies per flask, respectively). These data argue for a direct role of the protein instead of the RNA.

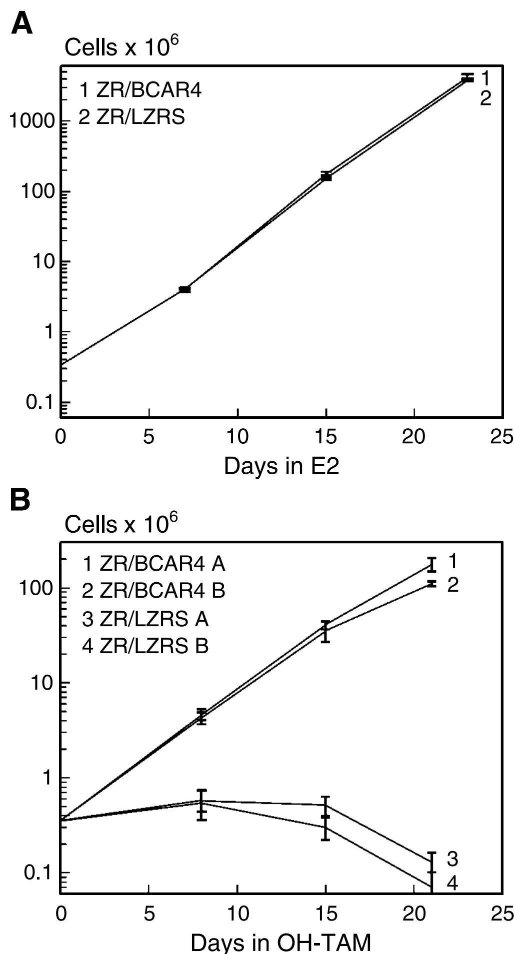

FIGURE 2. *LOC400500/BCAR4* induces antiestrogen-resistant proliferation. Independent pools of ZR-75-1 cells, infected with virus from the expression vector without cDNA insert (ZR/LZRS) or containing *LOC400500/BCAR4* (ZR/BCAR4), were plated in medium containing E_2 or OH-TAM in triplicate. At the time points indicated, the cells were counted and replated at the initial density. Cumulative cell numbers ± 95% confidence interval are presented over a 21-day culture period. **A.** proliferation curves of the cells in medium containing E_2. **B.** proliferation curves of the cells in duplicate in medium containing OH-TAM.

A soft agar assay was used to determine anchorage-independent transformation of ZR/BCAR4 cells *in vitro*. Parental ZR-75-1 cells are only capable of anchorage-independent proliferation in the presence of estradiol. In the absence of estradiol, colony formation is almost fully abrogated. ZR-75-1 vector control and ZR/BCAR4 cells (1×10^5) were plated in bovine calf serum–supplemented soft agar in duplicate. In ZR-BCAR4 cell cultures, colonies appeared within 10 days and were counted after 5 weeks. Large numbers of colonies were observed for ZR/BCAR4 cells (16,800 per 1×10^5) compared with ZR-75-1 vector control cells (45 per 1×10^5; Fig. 3).

Discussion

In this study, we identified seven genes involved in antiestrogen-resistant proliferation using *in vitro* retroviral transfer of cDNA expression libraries. Our breast cancer cell model for antiestrogen resistance has previously proven to be almost background-free, allowing the identification of genes involved in tamoxifen resistance using insertion mutagenesis (4). Random insertion of retroviruses in the cellular genome causes local disturbance of the genome structure (13) and may affect expression of adjacent genes (14, 15). Infection of ZR-75-1 cells resulted in the identification of seven BCAR genes and an additional seven common viral integration sites (5-7).[3] The frequency of retrovirally induced antiestrogen-resistant colonies was $\sim 5 \times 10^{-7}$ (4), and the probability of activating one of the BCAR genes most frequently found in our study was at least 10-fold lower. Our current experiments with retroviral transduction of cDNA libraries derived from human placenta, human brain, and mouse embryo proved to be 40 times more efficient in inducing tamoxifen resistance than insertion mutagenesis. Furthermore, identifying the genes responsible was less time consuming. On the other hand, the nature of the cDNAs identified in the screens is strongly dependent on the cDNA library used, whereas insertion mutagenesis is an almost random process. The contribution of insertion mutagenesis events in this study can be ignored in view of its very low frequency and the application of our stringent selection criteria, as detailed in the study outline and Table 1. Hence, this strategy obviates the need for a second round of selection involving replication-competent amphotropic viruses or time-consuming construction of suitable expression vectors.

[3] In preparation.

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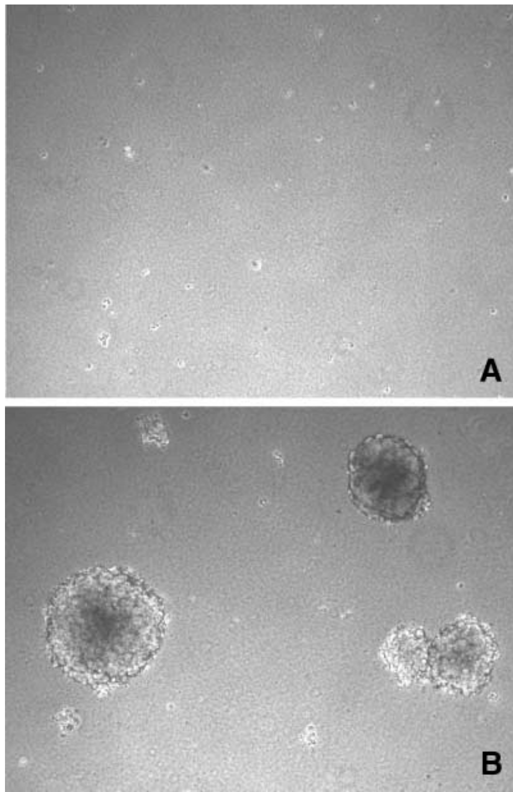

FIGURE 3. LOC400500/BCAR4 induces anchorage-independent transformation. A total of 1 × 10^5 ZR-75-1 vector control cells (ZR/LZRS) and 1 × 10^5 ZR-75-1 cells containing LOC400500/BCAR4 (ZR/BCAR4) were plated in bovine calf serum–supplemented soft agar in duplicate. Representative light microscopy photographs of flasks with ZR/LZRS (**A**) or ZR/BCAR4 (**B**) cells after 5 weeks.

Of the 73 genes identified in our current screens, 7 fulfilled both selection criteria and are thus considered to be responsible for antiestrogen resistance. Two of these genes (*EGFR* and *LOC400500/BCAR4*) have been confirmed to cause tamoxifen resistance in ZR-75-1 cells by gene transfer experiments (ref. 16 and this article). Four of these genes are expressed at extremely low levels (*EGFR*) or are undetectable (*PDGFRA, PDGFRB*, and *LOC400500/BCAR4*) in the parental ZR-75-1 cells (17).[4] Thus, the infected cells acquired a novel function essential for tamoxifen-resistant proliferation. Eighteen of the 73 genes complied only with the first criterion (Fig. 1; Table 1) and are considered to be interesting candidates. Formal prove of their role in tamoxifen resistance may be obtained in additional cDNA library transduction experiments or by individual gene transfer experiments.

The majority of the genes identified in this study are involved in RTK signaling. We identified four full-length RTKs: mouse *Pdgfra,* both human and mouse *PDGFRB,* human *CSF1R,* and human *EGFR,* which have been classified previously into two subfamilies based on common structural features (18). PDGFRA, PDGFRB, and *CSF1R* are members of the PDGFR RTK subfamily, which also encompasses KIT and the FGF receptors. Previous studies have shown the presence of *PDGFRB* mRNA in breast cancer cells (19). Aberrant expression of *CSF1R* has also been documented in a variety of malignancies, including breast cancer (reviewed by ref. 20). In mice bearing human breast cancer xenografts and treated with small interfering RNAs directed specifically against the *CSF1R* mRNA, tumor growth was suppressed by 40% to 50% and mouse survival increased (21).

EGFR is part of a RTK subfamily, including ERBB2, which is also among our list of candidate genes (Table 1), ERBB3, and ERBB4. EGFR has been reported previously to play an important role in normal development, differentiation, and cell proliferation (reviewed by ref. 22). High levels of EGFR promote antiestrogen-resistant proliferation in breast cancer cells and are associated with failure of tamoxifen treatment (16, 23). However, because expression of EGFR is inversely related to expression of ERα in breast cancers (24, 25), it is unlikely that EGFR plays a dominant role in antiestrogen resistance in ER-positive breast cancers.

We also identified the EGF-like ligand NRG1, which interacts with ERBB3 and ERBB4 receptors, leading to formation of ERBB homodimers or heterodimers (often including ERBB2). In our screens using both a human brain and a mouse embryo cDNA library, we identified the NRG1 sensory and motor neuron-derived factor isoform, which shares only the EGF-like domain with other NRG1 isoforms (26). High expression levels of NRG1 have been shown to cause progression of MCF-7 breast cancer cells to an estrogen-independent and antiestrogen-resistant state, which was later mimicked in a transgenic mice model (27).

FGF17, a ligand for the FGF receptor subfamily, was identified as well. Among known FGF family members, the FGF17 protein is most similar to FGF8 (28). Together with FGF8 and FGF18, it forms a subfamily, having similar gene structures, overlapping patterns of expression, and receptor-binding specificities (29). Overexpression of FGF8 and FGF17 in NIH-3T3 cells results in a transforming and tumorigenic phenotype (30, 31). Furthermore, FGF8 expression is significantly higher in breast cancer and in prostate cancer than in nonmalignant tissues (32, 33). Although no data are available for FGF17 expression in breast cancer yet, Heer et al. (34) reported a significant increase in *FGF17* mRNA expression in high-grade prostate cancers compared with benign prostatic hypertrophic tissues.

In addition to these known genes, we identified *LOC400500*, a putative gene positioned at human chromosome 16p13.1. It was recovered from 65% of the tamoxifen-resistant cell colonies obtained after transduction with a human placenta cDNA library. The gene has been designated as *BCAR4*. Sequence similarity analyses showed that all *BCAR4* cDNAs contained the same open reading frame of 121 amino acids. The amino acid sequence shows no homology with other proteins yet nor typical conserved domains. The presence of a putative signal peptide sequence[5] suggests that BCAR4 is transported to the membrane. The presence of two putative transmembrane regions further supports this hypothesis. The only homologues

[4] Unpublished data.

[5] http://www.ebi.ac.uk/InterProScan/.

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of BCAR4 reported until now were found in chimpanzee (100% identical) and rhesus monkey (92% identical). Thus, this gene may have emerged during the primate evolution. Here, we showed that this novel identified gene causes antiestrogen resistance in ZR-75-1 cells. Furthermore, loss of antiestrogen resistance due to a frameshift mutation strongly suggests that expression of the small protein is responsible for this resistance. We also showed anchorage-independent transformation of BCAR4 cells in a soft agar assay. From serial analysis of gene expression data[6] and expressed sequence tag profiles,[7] it is clear that that *BCAR4* is expressed in placenta and embryo. In addition, analysis of our expression data (35) and published array data (36, 37) indicates that *BCAR4* expression is present in ~10% of breast cancers.

In our future studies, we will establish which genes identified *in vitro* are important in progression of breast cancer in patients. We have already documented that genes identified in a screen for tamoxifen resistance can have clinical relevance (38, 39). The results from our planned studies will enable us to select particular genes for further study and to identify key regulators susceptible to targeted therapy.

In conclusion, retroviral transfer of cDNA libraries to human breast cancer cells is an efficient method for identifying genes involved in antiestrogen resistance *in vitro*. Six of seven genes that were identified in multiple resistant cell colonies are part of the mitogen-activated protein kinase pathway. This suggests that mitogen-activated protein kinase signaling may play an important role in tamoxifen resistance in human breast cancer. With the recent development of new tyrosine kinase inhibitors and antireceptor monoclonal antibodies, other genes involved in RTK signaling besides *EGFR* and *ERBB2* may represent valuable targets for therapy of tamoxifen-resistant breast cancer (40).

Materials and Methods

Cell Lines and Culture Conditions

ZR-75-1 cells (41) were cultured in RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 10% heat-inactivated bovine calf serum (standard medium; Hyclone, Logan, UT) and supplemented with 1 nmol/L E_2 (Sigma-Aldrich, St. Louis, MO) as described previously (16).

Cell colonies resistant to OH-TAM (Sigma-Aldrich) were picked and expanded in RPMI 1640 supplemented with 10% bovine calf serum and 10% conditioned medium of CRIP cells (mouse fibroblast cell line) and 1 μmol/L OH-TAM (4).

Transduction of Retroviral cDNA Libraries into ZR-75-1 Cells

Retroviral transduction experiments were done using retroviral cDNA expression libraries derived from human placenta, human brain, and mouse embryo (Clontech, Palo Alto, CA). Retroviral particles were produced by transient transfection of Phoenix-Ampho packaging cells (42) using FuGENE 6 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Cell-free supernatants containing viral particles were harvested after 2 and 3 days and stored at −80°C. In total, 38 flasks with ZR-75-1 cells were incubated with pLIB-cDNA virus in the presence of 4 μg/mL polybrene (Sigma-Aldrich) and 1 nmol/L E_2 to stimulate proliferation and integration of the retroviruses. To determine the infection frequency with fluorescence-activated cell sorting or fluorescence microscope analysis, cells were infected with a mixture of viral supernatant containing pLIB-EGFP (Clontech) together with viral supernatant of the placenta cDNA library. Alternatively, 1% of pLIB-EGFP construct was mixed with the library cDNA plasmids before production of the viral particles. Three days after infection, the cells were trypsinized and resuspended. The cells were plated in 75 cm^2 flasks (4×10^6 per flask) in medium containing 1 μmol/L OH-TAM. Within 6 weeks of plating, OH-TAM-resistant colonies were picked and transferred to 96-well or 48-well plates. Subsequently, the colonies were expanded for further characterization. Genomic DNA was isolated using NaCl extraction procedures as described by Miller et al. (43).

Analysis of Proviral cDNA Inserts

Integrated cDNAs were retrieved from genomic DNA by PCR using primers located in pLIB adjacent to the cDNA cloning site (44). PCR was done using the Expand High Fidelity PCR System (Roche) and the Expand Long Template PCR System (Roche) according to the manufacturer's instructions. PCR products containing multiple fragments were separated on and purified from 0.7% agarose gels using QIAquick gel extraction kits (Qiagen, Hilden, Germany). Sequence analysis was done on a LI-COR sequencer (LI-COR, Inc., Lincoln, NE) using Thermo Sequenase DYEnamic direct cycle sequencing kits (Amersham plc, Buckinghamshire, United Kingdom) or on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) using ABI Prism BigDye Terminators v3.0 cycle sequencing kits (Applied Biosystems) according to the protocols of the manufacturers. The cDNAs were identified by sequence similarity searches using Basic Local Alignment Search Tools.[8]

Expression Constructs and Proliferation Assays

A cDNA, containing the predicted coding region of *LOC400500,* isolated from one of the resistant cell lines, was cloned into the LZRS-IRES-Neo expression vector (45) of which viral particles were produced. ZR-75-1 cells were infected in duplicate with retroviral particles containing either the LOC400500 expression construct (ZR/BCAR4) or the LZRS-IRES-Neo expression vector without insert (ZR/LZRS). The cells were cultured in E_2-containing medium, and after 2 days, selection with G418 (Invitrogen) was started. G418-resistant cell colonies were pooled and expanded. Pools of ZR/BCAR4 and ZR/LZRS cells were harvested by trypsinization and counted with a Coulter Z1 cell counter (Coulter Electronics Ltd., Luton, United Kingdom). Subsequently, 3.5×10^5 cells were plated in triplicate in 25 cm^2 tissue culture flasks. Experimental medium containing 1 nmol/L E_2 or 1 μmol/L OH-TAM was changed twice weekly. Cells were trypsinized, counted, and replated in fresh medium at the initial density once weekly.

[6] http://bioinfo.amc.uva.nl/HTMseq/.
[7] http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.24611.
[8] http://www.ncbi.nlm.nih.gov/BLAST/.

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A frameshift construct of *LOC400500* was produced by inserting a thymine directly following the codon for amino acid 4. We did this site-specific mutagenesis with mutated PCR primers, and the resulting construct was sequence verified. ZR-75-1 cells were infected in duplicate with retroviruses containing the frameshift construct (BCAR4-fs). ZR/BCAR4 and ZR/LZRS cells were used as control cultures. The cells were cultured in E_2-containing medium and trypsinized and resuspended after 2 days. Five percent of the cells were plated in medium containing 1 μmol/L OH-TAM. Colonies of proliferating cells were counted after 15 days. To establish whether the infection was successful, another 5% of the cells were plated in 25 cm^2 flasks in E_2-containing medium. After 2 days, G418 selection was started and colonies of proliferating cells were counted 6 days after start of selection.

To determine *in vitro* anchorage-independent transformation of ZR/BCAR4 cells, a soft agar assay was done. ZR/LZRS and ZR/BCAR4 cells (1×10^5) were plated in duplicate in 0.3% soft agar with 10% bovine calf serum in RPMI 1640 culture medium on 0.6% base agar layers containing the same ingredients. Colonies were counted after 5 weeks.

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