

Identification of eight genes that are potentially involved in tamoxifen sensitivity in breast cancer cells

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ABSTRACT

Although the antiestrogen agent tamoxifen has long been used to treat women with hormone receptor positive invasive breast carcinoma, the mechanisms of its action and acquired resistance to tamoxifen during treatment are largely unknown. A number of studies have revealed that over-activation of some signaling pathways can cause tamoxifen resistance; however, very little information is available regarding the genes whose loss-of-function alternation contribute to tamoxifen resistance. Here we used a forward genetic approach *in vitro* to generate tamoxifen resistant cells from the tamoxifen sensitive breast cancer cell line ZR-75-1, and further identified the disrupted gene in different tamoxifen resistant clones. Retinol binding protein 7, DNA polymerase-transactivated protein 3, γ -glutamyltransferase-like activity 1, slit-robo RhoGTPase-activating protein, tetraspan NET-4, HSPC194, amiloride-sensitive epithelial sodium channel gene, and *Notch2*, were the eight mutated genes identified in different tamoxifen resistant clones, suggesting their requirement for tamoxifen sensitivity in ZR-75-1 cells. Since the functions of these genes are not related to each other, it suggests that multiple pathways can influence tamoxifen sensitivity in breast cancer cells.

Keywords: tamoxifen, breast cancer, gene identification.

INTRODUCTION

Tamoxifen is one of the most widely used drugs in the treatment of estrogen-receptor α (ER)-positive breast cancer. Used for over twenty years, tamoxifen has been shown to greatly prolong disease-free survival and induce remission in over half of patients with estrogen receptor positive metastatic disease [1]. In addition to being used as a treatment for breast cancer, tamoxifen has also been shown to be a preventative agent for hormone-dependent breast cancer and it is thought that a significant reduction in breast cancer mortality during the last decade can be contributed to the widespread use of tamoxifen [2]. Despite the obvious benefits of tamoxifen treatment, almost all patients with metastatic disease, and up to 40% of those receiving adjuvant tamoxifen, experience a relapse due to the development of drug-resistance.

It is believed that tamoxifen-resistance results from genetic alterations within tumor cells; however, the molecular mechanisms of tamoxifen resistance are largely

unknown. A number of cellular changes have been suggested to lead to tamoxifen-resistance. The loss of the ER could confer resistance since the effects of tamoxifen are primarily mediated through the ER [1]. Mutations of the ER genes may lead to a functionally negative ER phenotype without loss of ER expression, which also can result in tamoxifen resistance [3]. Altered expression of ER, may also lead to tamoxifen resistance [4]. Co-activator and repressor proteins have important roles in mediating transcriptional activation by the ER, and altered patterns of co-regulator expression may contribute to a tamoxifen resistant phenotype [5]. In addition to alterations at the ER level, growth factor and other signaling pathways may up-regulate and stimulate growth independently of the ER, or communicate via cross-talk with the ER, thus affecting cell growth and patterns of resistance [6].

The ER can be phosphorylated by the mitogen activated protein kinase family member ERK1 (extracellular signal-regulated kinase 1) and ERK2, both components of the growth signaling pathway. Phosphorylation enhances the sensitivity of the ER to the ligand and potentially leads to ligand-independent activation [7]. Furthermore, ribosomal S6 kinase (RSK), a downstream target of ERK1 and ERK2, can also phosphorylate the ER [8]. In addition to activating

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the ER directly, kinase-mediated growth factor signaling may also modulate ER activity indirectly by enhancing the activity of co-activators and attenuating co-repressor activity [9]. Therefore, it is possible that cross-talk between ER and growth factor receptor pathways, such as the EGFR/HER2 family and insulin-like growth factor receptor (IGFR) family up-regulating growth factor signaling pathways during tamoxifen treatment, may lead to loss of estrogen-dependence and tamoxifen resistance. Other MAP kinases such as JNK (c-jun N-terminal kinase) and p38 may also be involved in tamoxifen resistance/sensitivity since the development of tamoxifen resistance in MCF7 cells is accompanied by increased AP-1 binding activity [10], and activation of the p38 MAPK pathway was seen in 4-hydroxytamoxifen treated cells [11]. Another pathway that may play a role in tamoxifen resistance is the PI3K-Akt-mTor pathway, which is known to be anti-apoptotic. This pathway appears to promote cell survival in tamoxifen treated cells [12].

The studies reviewed above show that the contribution of EGFR/HER2 signaling, ERK, JNK, p38 and PI3K pathways in tamoxifen resistance is by no means certain [6]. Tamoxifen resistance is likely to be a complex process that involves numerous cellular pathways [6]. Current knowledge is far from adequate and intensive studies are needed. Here we used retroviral-mediated insertional mutagenesis to search for genes involved in tamoxifen sensitivity and have identified a number of genes whose mutation can lead to tamoxifen resistance *in vitro*. Since the identified genes are located in many different pathways, tamoxifen resistance seems to be caused by many different genetic alternations.

MATERIALS AND METHODS

Reagents

ZR-75-1 cells were obtained from the American Type Tissue Collection (Rockville, MD) and a single clone was isolated and used in the experiments. Phoenix cells were a kind gift from Gary Nolan (Stanford University, CA). 17β -estradiol, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), 4-hydroxy tamoxifen, chloroquine and polybrene were all purchased from Sigma (St. Louis, MO). ERK, p38 and JNK inhibitors, along with rapamycin and Z-Val-Ala-Asp-(OME)-fluoromethylketone (Z-VAD-FMK) were purchased from CalBiochem (San Diego, CA). G418 and Trizol were from Invitrogen (San Diego, CA).

Cell culture

ZR-75-1 cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% conditioned medium from 293 cells, 10 units/ml penicillin G sodium, 10 μ g/ml streptomycin sulfate, 292 μ g/ml L-glutamine, 0.1 mM MEM non-essential amino acids solution, 1 mM MEM sodium pyruvate, and 1 nM of 17β estradiol at 37°C and 5% CO₂. ZR-75-1 cells were detached using 0.25% trypsin solution and were passed through a

21-gauge needle. Phoenix cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 units/ml penicillin G sodium, 10 μ g/ml streptomycin sulfate, 292 μ g/ml L-glutamine, 0.1 mM MEM non-essential amino acids solution, and 1 mM MEM sodium pyruvate at 37°C and 5% CO₂. Phoenix cells were passed using 0.05% trypsin solution.

Treatments of ZR-75-1 cells

Selection of tamoxifen resistant ZR-75-1 mutants were performed using 1 μ M 4-hydroxy-tamoxifen for two to five weeks [13]. 50 μ M 4-hydroxy-tamoxifen was used to determine the effect of inhibition of ERK, JNK, p38, mTor pathways on tamoxifen-induced ZR-75-1 cell death in 24 h time-course experiments. The concentration used for the inhibitors of MEK (U0126), p38 (SB203580), JNK (SP600125), mTor (rapamycin), and caspase (zVAD) was 10 μ M, 10 μ M, 10 μ M, 45 nM, 20 μ M, respectively.

Cell death assays

Viability was assayed using both MTT assay and crystal violet assays. MTT assay was performed according to manufacturer's instructions [14]. Briefly, cells were washed twice with 1 \times PBS and incubated in 100 μ l of 0.5 mg/ml solution of MTT at 37°C for 30 min. After incubation, 100 μ l of MTT solubilization solution [10% Triton X-100 in acidic isopropanol (0.1 N HCl)] was added to each well to stop the reaction. Absorbance was read at 570 nm with background of 650 nm subtracted. Crystal violet assay was carried out as previously described [15]. Briefly, cells were washed twice with 1 \times PBS and 50 μ l of crystal violet solution (0.1% crystal violet, 10% methanol) and incubated at room temperature for 10 min. Cells were washed twice with 1 \times PBS, 100 μ l of a 50% glacial acetic acid solution was added, and cells were incubated at room temperature for 1 h. Absorbance was measured at 540 nm with background of 650 nm subtracted. Standard curves of absorbance and cell number were made for both methods and were used to calculate the percentage of viable cells.

In vitro mutagenesis

The retroviral plasmid pDisrup was prepared through cesium-chloride gradient ultra-centrifugation and transfected into the Phoenix retrovirus packaging cell line to produce recombinant virus. Virus expressing GFP (green fluorescent protein) was prepared in parallel and used to determine the relative virus titer. The titer that gave ~10% infection efficiency was used in the mutagenesis. Briefly, 4 \times 10⁶ Phoenix cells were seeded per 10 cm dish and cultured overnight. The cells were transfected with 15 μ g of pDisrup retroviral vector using calcium phosphate precipitation. The virus-containing medium was collected 36 h after transfection and filtered through a 0.45 μ M filter. A proper amount of virus-containing medium containing 4 μ g/ml polybrene was added to ZR-75-1 cells in 6-well plates and spun for 1 h at room temperature. After incubation overnight at 37°C and 5% CO₂, medium was changed to 17β estradiol-free medium and subjected to G418 and tamoxifen treatment.

Selection of tamoxifen-resistant ZR-75-1 mutants

Cells infected with pDisrup retrovirus were subjected to G418 selection (0.5 mg/ml) for 1 week followed by a combined treatment of G418 (0.5 mg/ml) and 4-hydroxy-tamoxifen (1 μ M) [13] for more than 2 weeks until individual clones could be isolated. Individual clones were picked up, seeded in 96-well plates, and expanded until

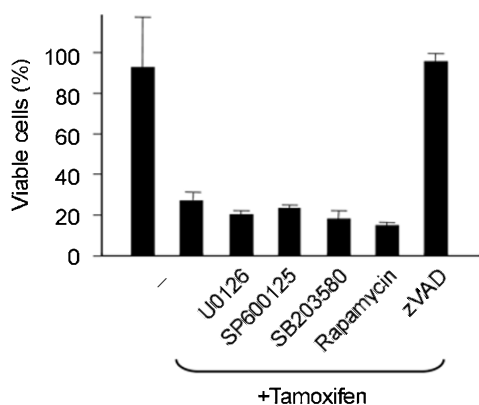


Fig. 1 Tamoxifen-induced ZR-75-1 cell death in the presence of different inhibitors. ZR-75-1 cells were pre-treated for 1 h with nothing (-), U0126 (10 μ M), SP600125 (10 μ M), SB203580 (10 μ M), rapamycin (45 μ M), or zVAD (20 μ M), and then treated with tamoxifen (50 μ M) for 24 h. Cell viability was determined by MTT assay. Data are expressed mean \pm SE as determined in triplicated samples.

confluent in 6-well plates. ZR-75-1 cells without infection of pDisrup virus were treated with 4-hydroxy-tamoxifen (1 μ M) in parallel to determine the basal level of spontaneous resistance.

Identification of mutated genes by 3'RACE

RNA was isolated from tamoxifen resistant clones in 6-well plates using Trizol extraction. Reverse-transcriptase PCR (Invitrogen) was performed using a 3'RACE CDS Primer (5'-AAGCAGTGG-TATCAACGCAGAGTAC(T)₃₀N₁N-3'). 3'RACE PCR was then performed on the cDNA using a universal primer mix [Long (4 μ M): 5'-CTAATACGACTCACTATAGGGCAAGCAGTGGT-ATCAACGCAGAGT-3']; Short (2 μ M): 5'-CTAATACGACTCACTATAGGGC-3'], and a *Neo* gene-specific primer. Nested PCR was performed using a nested *Neo* gene-specific primer, and a nested universal primer (5'-AAGCAGTGGTATCAACGCAGAGT-3'). PCR samples were then run on 1% agarose gel and DNA was extracted using Qiagen Qiaquick Gel Extraction Kit (Qiagen). DNA was sequenced using a *Neo* specific primer and sequences were blasted against the NCBI database.

RESULTS AND DISCUSSION

Effects of inhibiting the signaling pathways that have been implicated in tamoxifen sensitivity of ZR-75-1 cells

ZR-75-1 cells are ER positive and sensitive to tamoxifen-induced cell death. Since the up-regulation of ERK, JNK, PI3K-mTor activities have been implicated to lead to tamoxifen resistance [6], we tried to check whether inhibition of these pathways can sensitize tamoxifen-induced cell death in ZR-75-1. ZR-75-1 cells were treated with tamoxifen in the presence or absence of MEK inhibitor

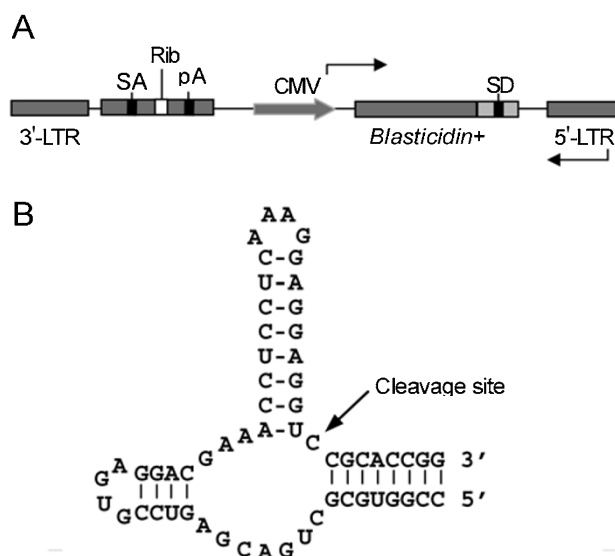


Fig. 2 (A) Diagram of the retroviral vector pDirup 8 used for gene knockout. Rib, ribozyme sequence; SD, splicing donor; SA, splicing acceptor; pA, poly A signal sequence; CMV, CMV promoter; *blastidin+*, blastidin resistant gene; (B) Sequence of the ribozyme in the vector.

U0126, JNK inhibitor SP600125, p38 inhibitor SB203580, or mTor inhibitor rapamycin. Caspase inhibitor zVAD was used as control to inhibit tamoxifen induced apoptosis. As shown in Fig. 1, zVAD inhibited tamoxifen-induced ZR-75-1 death, while inhibition of ERK, JNK, p38, or mTor all slightly enhanced tamoxifen-induced cell death as measured by MTT assay. Similar results were obtained with crystal violet assay (data not shown). Although inhibition of these pathways cannot significantly sensitize ZR-75-1 cells to tamoxifen induced cell death, our data does not exclude the possibility that over-activation of these pathways is still able to cause a resistance in ZR-75-1 cells to tamoxifen-induced cell death, as reported in other breast cancer cell lines [6]. Since sensitization of breast cancer cells to tamoxifen by targeting these pathways seems to have limitations, more effort should be given to identify more genes that are involved in tamoxifen induced cellular changes.

Mutagenesis of ZR-75-1 cells with retrovirus pDisrup

Studies on specific pathways have provided valuable information regarding gain-of-function alterations of some pathways in causing tamoxifen resistance [6]. However, there is very limited information regarding to loss-of-function mutations on the development of tamoxifen resistance. Generating tamoxifen resistant mutants from tamoxifen sensitive ZR-75-1 cells and identifying the mutations

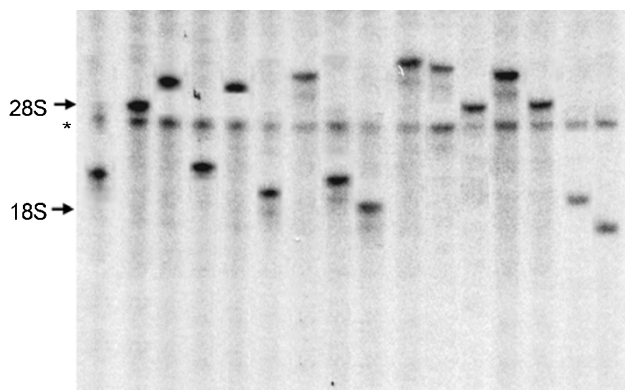


Fig. 3 Northern blotting of sixteen G418 resistant clones using ³²P-labeled double-strand *Neo* probe. Positions of 28S and 18S ribosomal RNA are indicated by arrows. A common band derived from viral genome by 5'-LTR is marked by an asterisk (*).

responsible for this phenotype change would allow us to identify genes whose function is required for tamoxifen sensitivity in breast cancer cells. Retroviral insertion can abolish gene expression or produce a truncated gene product depending upon the site of insertion. Truncation of the gene product might yield unpredictable results, since some truncated proteins might display dominant properties, causing either a gain- or loss-of-function. In order to produce gene deletion and avoid gene truncation, we incorporated a self-cleavage ribozyme sequence into the retroviral vector pDisrup 8 (Fig. 2A). The sequence of the self-cleavage ribozyme in the vector is shown in Fig. 2B. Any transcript containing this ribozyme will be destroyed because the mRNA will be self-cleaved and subsequently degraded. Previous studies have shown that this type of self-cleaving ribozymes results in a complete cleavage of RNA and in our experience, all of the transcript incorporating the ribozyme encoding sequence generated by a CMV promoter were destroyed (data not shown). In order to select the cells that have a viral insertion in a gene area, and to quickly find the insertion site, we adopted a poly A trapping strategy. Poly A trapping was selected over promoter trapping because the marker gene expression in poly A trapping does not depend on the activity of the endogenous promoter, and 3' rapid amplification of end of cDNA (RACE) performed after poly A trapping to identify the disrupted gene is much easier to apply than 5' RACE used after promoter trapping. Since the expression of *Neo* is dependent on the downstream poly-A signal sequence from the endogenous gene, G418 resistance was used to select the cells that had a viral insertion in a gene (either in an intron or exon). We termed this to be a functional insertion. To check the number of the functional

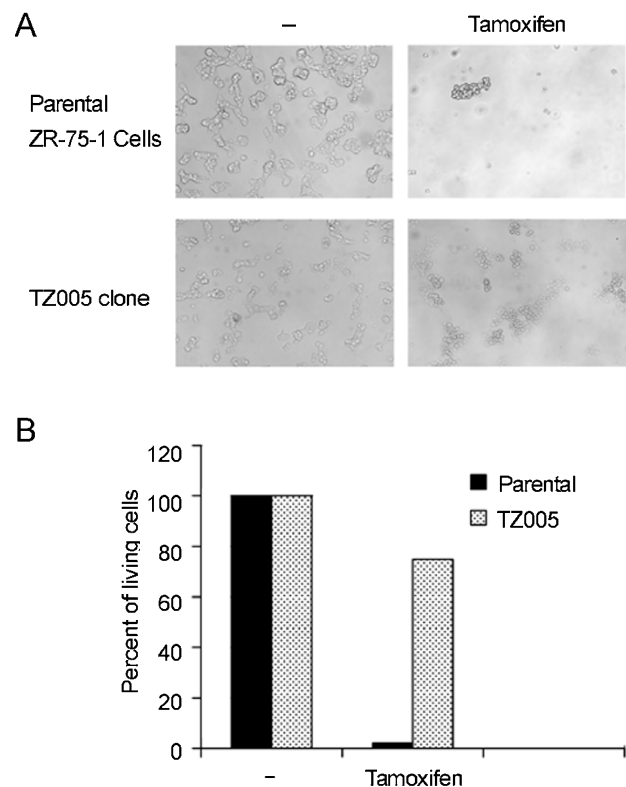


Fig. 4 (A) Tamoxifen resistance of a tamoxifen-resistant clone generated by pDisrup viral insertion. Parental and a representative tamoxifen resistant mutant clone were treated with tamoxifen for 24 h and then examined by phase contrast microscopy. (B) Percentage of living cells from both parental and representative tamoxifen resistant mutant clone before and after tamoxifen treatment.

insertions, we randomly picked up 16 G418-resistant clones from the L929 cells that were infected with pDisrup at a titer that can result in a ~10% infection efficiency. The *Neo* transcript was analyzed in these 16 clones by Northern blotting. Only one transcript was found in each of the clones indicating that only one functional insertion occurred in most of the G418-resistant clones (Fig. 3). This is crucial to the efficacy of this protocol because multiple gene disruptions will hinder the determination of which disrupted gene is responsible for the functional alteration. Because only ~2% of the genome encodes genes, statistically there is one functional insertion in every 50 insertions. So even if multiple virus integration occurs in a given cell, the functional insertion in most cases will still be one. This helps to explain the data observed in Fig. 3 that only one functional insertion was found in the clones we isolated.

We infected 10⁸ ZR-75-1 cells with the pDisrup retroviral vector at 10% infection efficiency and obtained ~5×10⁴ G418 resistant clones. The efficiency for generating the resistant clones is close to our estimation that 1% of the

cells infected with the virus will be G418 resistant (1/50 insertion occurred in a gene region and only one orientation of viral insertion in a gene region led to the expression of a G418 resistant gene). Thus, we obtained $\sim 5 \times 10^4$ mutant ZR-75-1 clones in which one allele of a gene has been disrupted.

Selection of tamoxifen-resistant ZR-75-1 mutants

Because some genes are haploid insufficient, single allele deletion of some genes should be able to lead to phenotype changes. Therefore, we treated the G418 resistant clones with tamoxifen to select tamoxifen-resistant mutants. Cell proliferation was stopped first, with cell death occurring thereafter. Sixty tamoxifen-resistant clones were obtained from the G418 resistant clones after three to six weeks of tamoxifen selection. In contrast, no visible clone was observed in tamoxifen-treated wildtype ZR-75-1 cells, suggesting that the tamoxifen-resistance in the survival clones most likely resulted from retrovirus-mediated mutagenesis. We picked up these sixty individual clones and transferred them into individual wells in 96-well plates. About half of the clones could be expanded. We then confirmed the tamoxifen resistance of the isolated clones by comparing their sensitivity to tamoxifen induced cell death with that of wildtype ZR-75-1 cells. The tamoxifen resistance of an isolated clone was shown in Fig. 4. All together, thirty clones exhibited a tamoxifen resistant phenotype.

Identification of the mutated genes

We expanded the thirty tamoxifen resistant clones to a sufficient number of cells for identification of the mutated genes. 3' RACE was performed using the RNA isolated from the tamoxifen-resistant clones, and we were able to amplify *Neo*-fusion mRNA from eighteen of the clones. Since there were a number of duplications, the total number of identified genes is eight. The representing sequences of the identified genes are shown in Fig. 5.

Sequence comparisons with genes in the GenBank database revealed that Tz002, Tz004, Tz005, Tz011, Tz051, Tz068, Tz160, Tz162 contain the cDNA sequences of retinol binding protein 7 (RBP7 protein), DNA polymerase-transactivated protein 3, γ -glutamyltransferase-like activity 1, slit-robo RhoGTPase-activating protein, tetraspan NET-4, HSPC194, amiloride-sensitive epithelial sodium channel gene, Notch2 gene, respectively. The *Neo* fused mRNA resulting from retroviral insertion was analyzed to determine the site of insertion in each clone. The viral insertion in Tz002 occurred in exon 2, 219 bp downstream of the start codon of *RBP7*; Tz004 occurred in exon 1, 206 bp downstream of the start codon of DNA polymerase-transactivated protein 3; Tz005 occurred intron 1, 4920 bp downstream of the start codon of γ -glutamyl-

transferase-like activity 1; Tz011 occurred in intron 1, 66 bp downstream of the start codon of slit-robo RhoGTPase-activating protein; Tz051 occurred in intron 4, 454 bp downstream of the start codon for tetraspan NET-4; Tz068 occurred in exon 2, 44 bp downstream of the start codon for HSPC194; Tz160 occurred in intron 1, 1988 bp downstream of the start codon for amiloride-sensitive epithelial sodium channel gene; and Tz162 occurred in exon 2, 80 bp downstream of the start codon of Notch2. None of these eight genes have previously been linked with tamoxifen signaling. Although the role of the identified genes in tamoxifen sensitivity still needs to be validated by other methods, the mutations identified in each clone are most likely responsible for the resistant phenotype because the mutation generated under our experimental conditions in most of the clones is one (Fig. 3) and no resistant clone was isolated from parallel treated wildtype cells.

Possible function of the identified genes in tamoxifen sensitivity

Since there is no information available regarding the function of DNA polymerase-transactivated protein 3 and HSPC194, we only discuss the potential function of the other six genes.

RBP7 (also named CRBP-IV) is a member of the cellular retinal-binding protein (CRBP) family and is involved in retinoic acid mediated cellular responses [16]. It is known that the growth of human breast tumor cells are regulated through signaling involving nuclear receptors of the steroid/thyroid/retinoid receptor gene family [17]. Retinoic acid receptors (RARs), members of the steroid/thyroid hormone receptor gene family, are ligand-dependent transcription factors which have *in vitro* and *in vivo* growth inhibitory activity against breast cancer cells [18]. It is possible that tamoxifen-induced ZR-75-1 cell death requires intact retinoid signaling and mutation of RBP7 impaired retinoid's function in ZR-75-1 cells. An example of the functional cross-talk between RAR and ER is that both antiestrogen and retinoid can also induce expression of PDCD4 (programmed cell death 4) in breast cancer cells [19]. Because RAR and ER share some co-activators, whether RBP7 has any direct role on ER is worthwhile to evaluate. RBP7 has been found in high abundance in mammary glands of mice and is known to complex with RBP4 to control transcription initiation [16]. It is also possible that aberrant RBP7 confers resistance to tamoxifen through alternation of transcription.

γ -glutamyl transferase-like activity 1 (GGTLA1) is known to exhibit acetyltransferase activity and γ -glutamyl transferase activity, both of which are important for many inflammatory responses [20]. GGTLA1 can convert leukotriene C(4) to leukotriene D(4), and leukotriene D(4)

TZ002

GTGGAGTTTGTCTGTGTGCTCAGGCTGGAGTGAATGGCGCATCTCGGCTCTCTGCATCCTCCGCTCCCGGGTTCAAGCGATTCTCTTGCC
TCGGCCTCCCGAGAAGCTGGGATTACGGGCATATGCCACCACGCTGGCTAATTTTGTATTTTGTAGTAGAGACCGGGTTTCTCCAAGTTGGTCAG
GCTGGTCTCACACTCCCAACCCAGGTGATCCGCCCGCTCGGCCTCCGAAAGTGTGGGATTACAGGCATGAGCCACTGTGCCCGCTGAAAGT
TTCTTTTTTGTTTTTTAACTGTCTCACCTGTACATATCTAAAACAGTGTATTTTATTTACTCTATGTTTATGTAAGAGTTTTAAATTTTGA
CAGTAGCTTAGATTTTCATTGGTAGAATATCAAGCTTGTTTAGGTTTTTAAG

TZ004

AGAGGACATCTGTGTGTTTTCCAGGCTGGACCTGAATGAACTTCTGGGCTCAATCAATCCTTCCCCTCAGCCTAACATTTACACTGGGAGCAG
AGATGCATTTGGAGTAGGTGATGATTTTGGAACTGCTTTAGAAGCAACATAGGTATCTGATTTTCATCAGGTCAAGCCATCAAAAATGACCAATAAA
TCTCTGACCTCTTAGAAGCAATTTGAAGACCCAAATTTGGCCATCATCAAAATTCGTGTTCATTCAAAATACACATTTCTGGAGAGCAAGGACA
ATCATTGTGCTGATGCTGCATTAATAATGCAGCTCTGAAGGTGACATCAAAACAGAACTCTCCAAATGACCTTGTGACTTATGACCCATTG
AAGACTTCATTAGAAGTACTAGTGGGCTGGGCATGGTGGCTCATGCCTGTAATCCAGCACTTTGGGAGGCAAGGCTGGCGGATCACCTGAGGT
CAGGAGTTTGAGCCAGCTGGCCAACATGGTGAATCCTGTCTCTATTAATAA

TZ005

ACCGAGTCTCGTTCTGTCAACCAGGCTGGAGTGCAGTGGCAGATCTCAGCTCAATGCAACCTCCACCTCCCGGGTTCAAGCGATTCTCTGCCT
CAACCTCCCGAGTACTGGGACTACAGGCACAGCCACCATGCCTGGTCCGAGACGGCGTACACACAGGCCCCCTCTGGGACACAGCCTGGACC
ACGTTCCAGGAAGAAGGGCTCTGGGCTGTGTTCTGGCCACGGTCTTGAGTCCCTCTGCACCTCTGGAGAGGAGAGAAAGCAGGTTACAGGG
GAGGCGAGCTCAGCCATCTCCAGGAGGGAGAGCCCTTGCAAGAAATGAG

TZ011

AAATTCGAGCTCAACTGGTAGAACAACAAAATGCCTGGAGCAGCAACCGAGATGCGAGTTCAGCTTCTCCAGGATCTGCAAGATTCTTCCGA
AAAAAGCTGAAATTTGAGACGGAAATTTCCCGAATCTAGAGAAGTTAGCAGAAAGGTTTCATGGCAAAAACAAGAGCCTAAGGATCATCAACA
ATACAAGAAAGACCAGAACCTGTTGTCTCCAGTGAATGCTGGTATTTGCTCTGAACCAAGTAAGGAGAGAAAGCAAAGACCATGCAACCTTGA
GTGACATCTATCTGAACAATGTGATTATGCGGTTATGTCAGATAAGTGAAGGATTTCTACCAGGATGTTTAAAAGAGCAAAGAGATTGCATTCAA
CTTCATGAGGATTTAATGAAGTTCCTAATGAGATTTATACGGTGTGAAAACCTACCCATGTATCATGCACAAGAATCCC

Tz051

CAGTGTCTCCGGGCTTTTGGAGCTGCATGATTGGTAACTAAATATTTACTTCAATTGCACANGATTCCAATGCAAGTCGCAGAGCGATGTGGCG
TTCATTCTCCTGCTGCATAAAGATCCCGCAGAAGATGTATCAACACTCAGTGTGGCTATGATGCCAGGCAAAAACAGAAAGTTGACCANCAG
ATTGTAATCTANACGAAAGGGTGTGTGCCCCAGTTTGAANAANTGGTGCAGGACAATTTAAANATCNTTGTGTTATTTTCATANGNATTTGNNT
GCTGCAGATATTTGGGATATGCTGGCCCAAAATTTGGNTAACGATATCAAACTGTGAGNNAGCTGGTAAACNCCCTGCAACCGNTNCTGCAAA
ACATGGACAGACNCACTTTTCCAGGACCTCCNGTGTGCNCAACAGNTCNTNNGACTGAANNGANCTAATNAACTGATGCAGCCTNCAATNTCCC
ATAATGNCACTTCCATTAAAGGANAGTAAAAACCCNGNAATGNTNNTACNCTCCACCAAAATAAC

Tz068

GTGCAGGCTGGGGTAGTCTCCTGTCTGGACAGAGAAGAGAAAAATGCAGGACACTGGCTCAGTAGTGCCTTTGCATTGGTTTGGCTTTGGCTAC
GCAGCACTGGTTGCTTCTGGTGGGATCATGGCTATGTAAGCAGGCGCGTGNCGTCCCTGGCTGNAGGGCTGNTCTTTGGCAGTCTAGCCGG
CCTGGGTGCTTACCAGCTGTCTCAGGATCAAGGAAACGTTNGGGTTTTCTAGCTACATCTGGTACCTTGGCTGGCATTATGGGAATGAGGTTCT
ACCACCTGGAATAATTCATGCCTGCAGGTTAATGTCAGGTGCCAGTTTGTCTGATGGTGCCTAAAGTTGGAGTTAGTATGTTCAACAGACCCCAT
TAGCAGAAGTCATGTTCCAGCTTAGACTGATG

Tz160

GACAGGACGTCACCCCTTTCTTAGTCCGTAACGCACCTTTGGACATGATGGAATAACGAAGGGCCTCCACCCACAGCATGACCAATCCCCAG
CATCCAAAATCGCACACATACCAATATGTGCACACACACTCACACCACACCCATAAAGATTTTGCACTCCTTGAAGGTACACTAACTCACCA
TTTNTATCATACTTATCCAGTGTGCCACAGTTACTGGCTTATATGCCTGTCTCTGTATCTTATTNATCTGTCTCCACACACAGCAACTAC
TGGCCTTCAATAAAGGGCTTATGAATTATTTCATGAATCATTGTCANGNGCCTACCTGNGTCTGCTTGAACAGGTGTCCCAAGGTGGNGGNTG
GCT

Tz162

GTCGAGATGGCTATGNACGCTGTGTAATGAAGGAATGTGTGTTACCTACCACAATGGCANAGGATACTGCAANTGTCCAGAAGGCTTCTTGGGG
GAATATTGTCAACATCGAGACCCCTGTGAGAAGAACCGCTGCCAGAATGGTGGACTTGTGTGGCCAGGCATGCTGGGAAAGCCACGTGCCG
ATGTGCCTCAGGTTTTACAGGAGAGGACTGCCAGTACTCGANATCTCATCCATGCTNTGTGTCTCGACCTNGCCTGAATGGCGGAAATGNCATA
TGCTCAGNCGGGGATACCTATTAGTTGNCCTGTCAAAGTCNGGTTTACANGGTAANGAAGNGCCAAT

Fig. 5 The sequences of cDNA deduced from the *Neo* fusion mRNA in tamoxifen resistant mutant clones.

has been shown to inhibit the growth of MCF-7 breast cancer cells [21]. Disruption of GGTLA1 may interfere the generation of lipid mediators like leukotriene D(4) and thereby promote breast cancer cell growth and lead to tamoxifen resistance.

Slit-Robo RhoGTPase-activating protein (SRGAP1) belongs to a novel family of Rho GTPase activating proteins (GAPs) and interacts with the intracellular domain of roundabout (Robo) [22]. The slit protein guides neuronal and leukocyte migration through the transmembrane

receptor Robo. Members of Rho GTPase family are known to play key roles in regulating various biological activities including actin organization, focal complex/adhesion assembly, cell motility, cell polarity, cell-cycle progression and gene transcription [22]. Some Rho GTPases are known to be required for breast cancer cell metastasis and contribute to malignant phenotypes of breast cancer cells [23], and our data suggest that the GAP protein SRGAP1 is involved in tamoxifen induced cellular responses.

Tetraspan NET4 (Tm4sf9) is a member of the tetraspanin

superfamily which has been implicated in such biological activities as cell motility, metastasis, cell proliferation and differentiation [24]. The tetraspans are components of large molecular complexes that include non-tetraspan molecules, in particular integrins. The tetraspanins are molecules with four transmembrane regions and, by interacting with each other, the tetraspanins are thought to generate a network of molecular interactions, the tetraspanin web. A recent report shows that tetraspanins interact with cholesterol, suggesting possible interaction of tetraspans with extracellular ligands [25]. It is difficult to predict what the role of Tm4sf9 is in tamoxifen sensitivity at this moment; however, because of the involvement of tetraspanin superfamily members in cell motility and proliferation, Tm4sf9 may function in these processes to interfere with tamoxifen induced cellular changes.

The amiloride-sensitive epithelial sodium channel (ENaC) is a member of the degenerin/ENaC family of ion channels and regulates fluid and electrolyte absorption across a number of epithelia [26]. ENaC are developmentally regulated, selectively expressed, and variously up-regulated by steroid hormones and estrogen [27]. Since ENaC is of fundamental importance in controlling sodium balance, it may be associated with many cellular processes, and its association with cell death has been reported in *C. elegans* [28].

Notch2 is a member of the Notch family, a conserved Type 1 transmembrane receptor [29]. Notch proteins modulate differentiation, proliferation, and apoptotic programs in response to extracellular ligands expressed on neighboring cells [30]. Ligand-mediated stimulation of Notch causes the proteolytic release of the intracellular domain (NotchIC), which then passes into the nucleus where it activates transcription of CBF1 responsive genes [31]. Deregulation of this pathway by over expression of a constitutively activated form of Notch not only diverts cell fate decisions but is also tumorigenic [32]. Notch2 has been shown to be involved in cell cycle arrest and cell growth, and, interestingly, can induce cyclin D1 transcription and CDK2 activity [31]. It is possible that Notch2 is involved in tamoxifen-induced cell arrest and that disruption of Notch2 in ZR-75-1 cells can relieve this block and render cells tamoxifen resistant.

Recently, evidence has implicated epidermal growth factor receptor (EGFR) signaling, along with Akt1 and Akt2, members of the PI3 kinase (PI3K) signaling pathway, as contributors to tamoxifen resistance in breast cancer cells [33, 34]. Overexpression of EGFR has been shown to confer antiestrogen resistance in estrogen-receptor positive breast cancer cells and Akt is known to induce estrogen-independent transcription of the estrogen receptor. Interestingly, both retinol-binding proteins and notch proteins can

regulate or are regulated by EGFR and/or PI3K. A recent study has shown that cellular retinol-binding protein-I (CRBPI) can inhibit the PI3K/Akt signaling pathway in a retinoic acid receptor-dependent manner, while several other studies have provided evidence for PI3K and EGFR dependent activation of notch signaling in tumorigenesis [35–37].

It has been proposed that tamoxifen resistance can result from many different mechanisms, and different approaches such as proteome and microarray analysis have been used to identify potentially important components that contribute to acquired antiestrogen resistance with some progress. Our approach of forward genetic screening appears to be effective in searching for new components that participate tamoxifen sensitivity. The potential importance of the eight unrelated genes in tamoxifen sensitivity supports the idea that the development of tamoxifen resistance is a complex process. Our data suggest that tamoxifen-induced ZR-75-1 cell death may require a number of pathways such as Notch2, slit-Robo, lipid mediator leukotriene D(4), and signaling of other nuclear receptors. Future work should focus on the validation of identified genes and exploration of the functional relationship between the identified genes and the genes that are known to be potentially involved in the development of tamoxifen resistance as the identification of genes that are required for tamoxifen sensitivity may provide new therapeutic targets for anti-hormone resistant tumors.

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