Supplementary Appendix

Methods

Cell lines

Human breast cancer cell line MCF7 derived cell lines MCF7aro, LTEDaro, LET-R, HER2aro, AKTaro were generated in this laboratory and were characterized and described previously [1-3]. MCF7aro was cultured in minimal Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin-streptomycin, and 0.1 mg/mL G418. AI-resistant cell lines LET-R and LTEDaro were maintained in phenol red–free MEM containing 10% charcoal/dextran-treated FBS with identical supplements as parental MCF7aro cells. LET-R was continuously cultured in the presence of 200 nM letrozole and 1 nM testosterone. HER2aro and AKTaro were cultured in the same medium as MCF7aro plus 50 µg/mL hygromycin B.

Western blotting

Western blotting was performed as previously described [4]. In brief, cells were lysed in RIPA buffer (Cell Signaling) on ice for 5 min and then sonicated for 60 s. The protein concentration was determined by protein assay kit (Bio-Rad), and the samples were separated by 10% SDS-polyacrylamide gel electrophoresis. After probing with a primary antibody, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody. Finally, signal intensity was determined with the SuperSignal West Pico Chemiluminescent (Thermo Scientific) substrate visualization. Relative expression of proteins was normalized against the internal control GAPDH.

Real-time PCR

Total RNA was extracted from cells using the TRIzol reagent (Invitrogen) and quantified with a Nanodrop spectrophotometer. For real time PCR of PDCD4, reverse transcription was performed with SuperScript VILO cDNA synthesis kit (Invitrogen) from 2.5 µg total RNA. For real time PCR of miR-21, reverse transcription was performed with qScript microRNA cDNA synthesis kit (Quanta Biosciences) from 1 µg total RNA. Real time PCR was performed using iQ5 multicolor real-time PCR detection system (Bio-Rad). The human PDCD4 gene was amplified using the forward primer 5′-ACAGGTGTATGATGTGGAGGA-3′ and the reverse primer 5′-TTCTCAAATGCCCTTTCATCCAA-3′ (PrimerBank ID 313760536c3) [5]. The ACTB (β-actin) gene was amplified using the forward primer 5′-CACCAACTGGGACGACAT-3′ and the reverse primer 5′- GCACAGCCTGGATAGCAAC-3′. The miR-21 and U6 RNA were amplified using miR-21 or U6 RNA specific primer in conjunction with the universal

PCR primer, all purchased from Quanta Biosciences. The real time PCR was established with the PerfeCTa SYBR Green SuperMix (Quanta Biosciences). The PCR results were normalized with β-actin (for PDCD4) or U6 RNA (for miR-21) as an internal control and then expressed as relative expression compared with reference samples. Each experiment was performed in triplicate. The data are expressed as means ± standard errors (SE).

Transfection

Transfection of LTEDaro and HER2aro cells with control siRNA/siRNA against MAPK, or control inhibitor/anti-miR-21 inhibitor was performed using the siPORT NeoFX transfection agent (Ambion) according to the manufacturer's protocol. Transfection of LTEDaro cells with estrogen responsive element (ERE) reporter plasmid was performed using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfection of LET-R and LTEDaro cells with pMG-H2-PDCD4 expression construct or empty vector was performed using the JetPRIME (Polyplus-Transfection) according to the manufacturer's protocol. For immunoblotting, transfection was performed in 60-mm dishes. For luciferase reporter assay, 24-well plates were used. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 96-well plates were used.

Luciferase reporter assay

LTEDaro cells were seeded and cultured in phenol red–free MEM containing 10% charcoal/dextran-treated overnight, and then transiently transfected with pGL3 (ERE)$_3$ reporter plasmid using the Lipofectamine 2000 (Invitrogen). Four hours after transfection, the cells were treated with either DMSO or 100 nM fulvestrant. After 24 hours of incubation, the cells were lysed with passive lysis buffer (Promega). The luciferase activity of cell lysates was assayed using luciferase assay substrate (Promega), and the protein concentration was measured using the Bio-Rad protein assay. The relative luciferase activity was calculated by dividing the luciferase activity by the protein concentration. Each transfection was performed using four replicates. The data are expressed as means ± SE.

Cell proliferation assay

Cell proliferation was measured by an MTT assay. At the indicated time, the medium in each well in 96-well plates was removed from the cells and replaced with 0.1 mL of fresh phenol red–free medium containing 0.5 mg/mL MTT, and then the cells were incubated at 37°C for 1 hour. After discarding the medium, the formazan dye trapped in the living cells was dissolved in 0.1 mL of DMSO and absorbance was measured at 570 nm with a SpectraMax M5 plate reader (Molecular Devices). Each experiment was performed using at least three replicates. The data are expressed as means ± SE.

Gene expression analysis

PDCD4 differential expression analysis was performed in The Cancer Genome Atlas (TCGA) cohort in breast cancer patients with ER and HER2 status [6]. The differential expression *p*-values were determined via t-test using R [7] (http://www.R-project.org/). Disease-free survival analyses according to PDCD4 expression were determined in two independent prospective cohorts in breast cancer patients with ER status and disease-free survival outcomes. In the Ivshina et al. study [8], the gene expression was profiled with 347 primary invasive breast tumors using Affymetrix U133A&B GeneChips microarray. We downloaded the dataset from the NCBI Gene Expression Omnibus (GEO) database (GSE4922 & GSE1456), and performed quantile normalization. In the Wang et al. study [9], the gene expression was profiled with 286 primary invasive breast tumors using Affymetrix U133A GeneChips microarray. We downloaded the dataset from the NCBI GEO database (GSE2034), and performed quantile normalization. The high *PDCD4* expression and low *PDCD4* expression groups were segregated based on median expression values. Kaplan-Meier survival analysis was used to determine the disease-free survival differences between the high *PDCD4* expression and low *PDCD4* expression groups among patients with ER-positive breast cancer in two cohorts, and visualized by Kaplan-Meier plots and compared using Cox regression analysis, with *p*-values calculated by log-rank test using the survival package in R [7]. *PDCD4* differential expression analysis was also performed in patients of the Ivshina cohort according to Elston histological grade of breast tumors [8].

Figure S1: Gene expression analysis in patients with low grade (Elston grade1, n = 68) or high grade (Elston grade 3, n = 55) in the Ivshina cohort. Differential expression analysis for PDCD4 shows a lower level of PDCD4 in patients with high grade (Elston grade 3) tumors than in patients with low grade (Elston grade1) tumors.
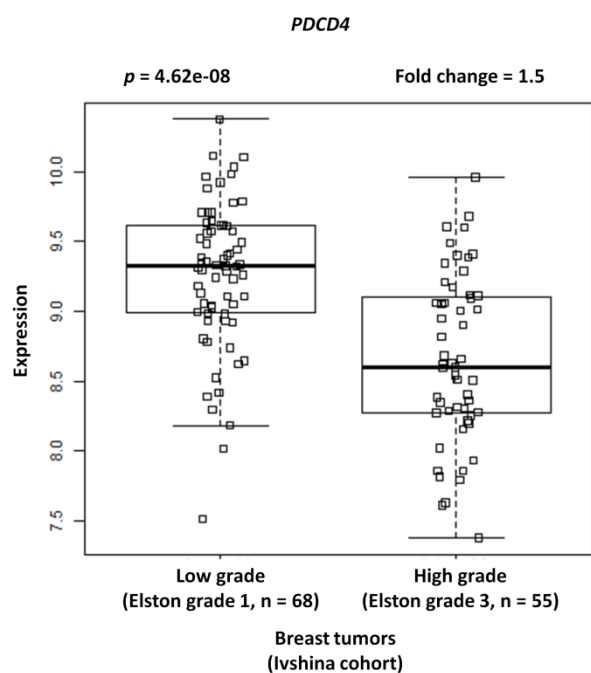
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