Supporting Information

Loi et al. 10.1073/pnas.1222251110

SI Materials and Methods

Gene-Expression Data and Survival Analyses. We retrieved all clinical and gene expression data of previously reported microarray datasets (Table S1). To ensure comparability of expression values across multiple data sets, *ESR1*, *ERBB2*, and *AURKA* gene expression values were rescaled before applying the subtype classifier as in ref. 1 (we used SCMOD1 classifier, which is referred to as SCM, Subtype Classification Model, in the present study). Our rescaling approach is implemented and fully documented in our R/Bioconductor package *genefu* v1.5.2 [see function 'rescale' (1)]. The TNBC identified using the SCM classifier is highly concordant with the "basal-like" subtype using the PAM50 classifier (2) and shows low *ESR1*, *PgR*, and *ERBB2* expression (Fig. S1). Differences in expression of CD73 according to subtype was examined using the Kruskal–Wallis test. Distant metastasis-free survival was the primary survival end-point, which is defined as the time elapsing between breast cancer diagnosis and date of local or systemic relapse, or death. When distant metastasis-free survival data were not reported, relapse-free survival information was used if available. For visualization, survival plots according to the CD73 tertiles were drawn using the Kaplan–Meier method, and the significance of the survival differences were evaluated using the log-rank *P*-test. In 137 cases, identification of subtype was not possible because of the absence of *AURKA*, *ESR1*, or *ERBB2* gene-expression information. These cases were included in "all patients" analyses. To assess correlation with clinical outcomes with just an anthracycline chemotherapy alone, we analyzed a cohort of breast cancer patients treated with preoperative epirubicin (a commonly used anthracycline) chemotherapy for four cycles before surgery in the setting of a clinical trial previously described (3). The clinical endpoint used was pathologic complete response (pCR) rates documented at surgery, or complete disappearance of invasive disease, which is an accepted surrogate for disease-free and overall survival in $ER^-/HER2^-$ breast cancer (4). CD73 levels were correlated with pCR as a continuous variable (i.e., to determine whether higher expression correlated with a higher chance of obtained pCR), using a receiver operating characteristic (ROC) curve, with the predictive ability assessed by calculating the area under the curve (AUC) together with 95% CI using the concordance index. Patients were negative for expression of the ER using immunohistochemistry and negative for *ERBB2* amplification detected by FISH.

CD73/CD39 Up-Regulation Assays. T47D, BT474, SKBR3 and MDA-MB-231 cells were cultured in DMEM (Wisent) 10% (vol/vol) FBS (Invitrogen). ZR75 cells were cultured in RPMI (Wisent) 10% FBS and MDA-MB-468 were cultured in DMEM/F12 (Invitrogen) 10% FBS. Human breast cancer lines were a generous gift from Sylvie Mader (Institute for Research in Immunology in Cancer, Montreal, Canada). LOX-1MV1 and A2058 cells (a generous gift from Karen Sheppard, Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia) were cultured in RPMI 10% (vol/vol) FCS with Hepes, glutamax, and pen-strep. RPMI-8226 cells (a generous gift from Ricky Johnstone, Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia) were cultured in RPMI 10% FCS with glutamax, and pen-strep. Kasumi-1 cells (a gift from Ricky Johnstone, Peter MacCallum Cancer Centre, East Melbourne, VIC, Australia) were cultured in RPMI 20% FCS with glutamax, nonessential amino acids, sodium pyruvate, Hepes, and pen-strep. For in vitro assays, cells were seeded for 24 h, then treated with chemotherapeutic drugs diluted in their respective culture media. After 48 h of treatment, cells were collected and stained for flow cytometry with PE-conjugated anti-human CD73 mAb (clone AD2; BD Bioscience) and APC-conjugated anti-human CD39 mAb (clone TU66; BD Bioscience), except for doxorubicin (DOX)-treated cells, which were stained with APC-conjugated anti-human CD73 (clone AD2) for breast cancer cells or purified anti-human CD73 mAb (clone 1D7, Abcam) followed by FITC-conjugated secondary antibody for melanoma and leukemia cells. Data were acquired using an LSR Fortessa (BD Biosciences) and analyzed using FlowJo software.

CD73 Expression in Vivo. Female nonobese diabetic (NOD)-SCID mice (JAX mice, The Jackson Laboratory) were injected subcutaneously with 10^6 MDA-MB-231 cells and treated at day 20 with an intratumoral injection of DOX (1 mM in 50 μL PBS) or PBS (three mice per group). Tumors were removes 48 h later for CD73 expression analysis. For IHC, tumors were embedded in OCT and snap-frozen. Sections were cut at 5 μm with a cryostat microtome, fixed in 100% (vol/vol) precooled (−20 °C) acetone, incubated with 1.5% (vol/vol) H_2O_2, and blocked for 30 min with protein block solution (Dako), incubated with anti-human CD73 mAb (clone 1D7; Abcam) for 1 h followed by biotin-conjugated secondary antibody and streptavidin-conjugated HRP (Dako). For immunoblotting, tumor lysates (20 μg of protein) were subjected to SDS/PAGE, and transferred onto nitrocellulose membranes (0.45 μm) (Bio-Rad), blocked 1 h in 5% (wt/vol) milk and incubated with anti-human CD73 mAb (1:1,000) clone 1D7 (Abcam) and anti-Actin (1:25,000) at 4 °C overnight. Detection was carried out using anti-mouse HRP-conjugated secondary antibody and chemiluminescence-based detection systems according to the manufacturer's recommendations (Thermo Scientific). For LI-COR imaging, membranes were incubated with anti-CD73 (1:2,000) and anti-GAPDH (1:5,000) at 4 °C overnight and proteins detected with fluorescence-conjugated anti-mouse or anti-rabbit antibodies (IR-Dye 800CW or IRDye 680RD; Li-COR) using Odyssey (Li-COR). Quantification of bands was done using Image studio 2.0 software (Li-COR).

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Table S1. Compendium of microarray datasets of unique breast cancer patients

Dataset	Microarray technology	Survival data	Treatment	No. of patients	No. of probes	Source	Reference
EXPO	Affymetrix HGU	NA	NA	353	54,675	GEO: GSE2109	1
VDX*	Affymetrix HGU	RFS, DMFS	Untreated	344	22,283	GEO: GSE2034/GSE5327	2, 3
NKI*	Agilent	RFS, DMFS, OS	Untreated, chemo	337	24,481	Rosetta Inpharmatics	4, 5
UCSF*	In-house cDNA	DNFS, RFS, OS	Untreated, chemo, hormonal	170	10,368	Authors' Web site	6, 7
STNO2*	In-house cDNA	RFS, OS	Untreated, chemo, hormonal	122	7,787	SMD	8
NCI*	In-house cDNA	RFS	Untreated, chemo, hormonal	99	6,878	Authors' Web site	9
MSK	Affymetrix HGU	DMFS	Heterogeneous	99	22,283	GEO: GSE2603	10
UPP*	Affymetrix HGU	RFS	Untreated, hormonal	251 (190)†	44,928	GEO: GSE3494	11
STK	Affymetrix HGU	RFS	Untreated, chemo, hormonal	159	44,928	GEO: GSE1456	12
UNT*	Affymetrix HGU	RFS, DMFS	Untreated	137 (94)†	44,928	GEO: GSE2990	13, 14
UNC4*	Agilent	RFS, OS	Heterogeneous	337	17,779	UNC DB	15
NCH	Agilent	DMFS, RFS, OS	Heterogeneous	135	17,086	AE: E-UCON-1	16
IGR2	Afymetrix	NA	Chemo	49	22,283	Journal Web site	17
CAL*	Affymetrix HGU	RFS, DMFS, OS	Chemo, hormonal	118	22,283	AE: E-TABM-158	18
TRANSBIG*	Affymetrix HGU	RFS, DMFS, OS	Untreated	198	22,283	GEO: GSE7390	19
DUKE	Affymetrix HGU95	OS	Heterogeneous	171	12,625	GEO: GSE3143	20
DUKE2	Affymetrix X3P	NA	Chemo	160	61,359	GEO: GSE6961	21
MAINZ*	Affymetrix HGU	DMFS	Untreated	200	22,283	GEO: GSE11121	22
LUND2	Swegene	NA	Hormonal	105	27,648	GEO: GSE5325	23
LUND	Swegene	NA	Heterogeneous	143	26,824	GEO: GSE5325	24
LUH	In-house cDNA	NA	Heterogeneous	58	3,389	Authors' Web site	25
FNCLCC	In-house cDNA	NA	Chemo	150	9,216	GEO: GSE7017	26
MDA	Affymetrix HGU	NA	Chemo	133 (3)†	22,283	MDACC DB	27
MDA3	Affymetrix HGU	NA	Chemo	45	22,283	MDACC DB	NA
MDA4	Affymetrix HGU	NA	Chemo	129 (64)†	22,283	MDACC DB	28, 29
MDA6	Affymetrix HGU	NA	Chemo	102 (6)†	22,283	MDACC DB	30
EMC2*	Affymetrix HGU	DMFS	Chemo	204	54,675	GEO: GSE12276	31
MUG	Operon	NA	Chemo	152	35,788	GEO: GSE10510	32
NCCS	Affymetrix HGU	NA	NA	196	22,283	GEO: GSE5364	33
MCCC	Illumina	NA	NA	75	48,701	GEO: GSE19177	34
KOO*	Affymetrix HGU95	NA	NA	88	48,701	Authors' Web site	35
EORTC10994	Affymetrix HGU	NA	Chemo	49	22,283	GEO: GSE1561	36
HLP	Illumina	NA	Chemo	53	48,701	AE: E-TABM-543	37
DFHCC*	Affymetrix HGU	DMFS	Heterogeneous	115	54,675	GEO: GSE19615	38
DFHCC2	Affymetrix HGU	NA	Chemo	84	54,675	GEO: GSE18864	39
DFHCC3	Affymetrix HGU	NA	Chemo	40 (33)†	54,675	GEO: GSE3744	40
DFHCC4*	Affymetrix HGU	NA	Untreated	129 (98)†	54,675	GEO: GSE5460	41
MAQC2	Affymetrix HGU	NA	Chemo	230	22,283	GEO: GSE20194	42
JBI	Affymetrix HGU	NA	NA	97	54,675	GEO: GSE20711	43
MGH	Arcturus	DMFS, RFS	Hormonal	60	22,575	GEO:GSE1378	44
TAM	Affymetrix HGU	DMFS, RFS	Hormonal	345 (242)†	44,928	GEO: GSE6532/GSE9195	45
MDA5	Affymetrix HGU	DMFS	Hormonal	298	22,283	GEO: GSE17705	46
VDX3	Affymetrix HGU	DMFS	Hormonal	136	22,283	GEO: GSE12093	47
TOP	Affymetrix HGU	pCR	Chemo	120	54,675	GEO: GSE16446	48

*Microarray datasets of unique breast cancer patients (6,209) used in this study were retrieved from journal or authors' Web sites, Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo/), ArrayExpress (AE; http://www.ebi.ac.uk/arrayexpress/), Stanford Microarray Database (SMD; http://smd.stanford.edu/), MD Anderson Cancer Center Microarray database (MDACC DB; http://bioinformatics.mdanderson.org/pubdata.html), University of North Carolina database (UNC DB; https://genome.unc.edu), and Rosetta Inpharmatics (www.rosettabio.com/). Each dataset was assigned a short acronym and an instance number if several datasets were published by the same institution or consortium: CAL: dataset of breast cancer patients from the University of California, San Francisco and the California Pacific Medical Center (United States); DFHCC: Dana-Farber Harvard Cancer Center (United States); DUKE: Duke University Hospital (United States); EMC: Erasmus Medical Center (The Netherlands); EORTC10994: Trial number 10994 from the European Organization for Research and Treatment of Cancer Breast Cancer (Europe); EXPO: Expression Project for Oncology, large dataset of microarray data published by the International Genomics Consortium (United States); FNCLCC: Fédération Nationale des Centres de Lutte contre le Cancer (France); HLP: University Hospital La Paz (Spain); JBI: Jules Bordet Institute (Belgium); KOO: Koo Foundation Sun Yat-Sen Cancer Centre (Taiwan); LUH: Lund University Hospital (Germany); LUND: Lund University Hospital (Sweden); MAINZ: Mainz hospital (Germany); MAQC: Microarray Quality Control Consortium (United States); MCCC: Peter MacCallum Cancer Centre (Australia); MDA: MD Anderson Cancer Center (United States); MGH: Massachusetts General Hospital (Boston, MA); MSK: Memorial Sloan-Kettering (United States); MUG: Medical University of Graz (Austria); NCCS: National Cancer Centre of Singapore (Singapore); NCH: Nottingham City Hospital (U.K.); NCI: National Cancer Institute (United States); NKI: National Kanker Instituut (The Netherlands); STK: Stockholm. Karolinska University Hospital (Sweden); STNO: Stanford/Norway (United States and Norway); TAM: tamoxifen-treated dataset collected by Jules Bordet Institute (Belgium); TOP: TOP trial initiated at the Jules Bordet Institute; TRANSBIG: dataset collected by the TransBIG consortium (Europe); UCSF: University of California, San Francisco; UNC: University of North Carolina (United States); UNT: cohort of untreated breast cancer patients from the Oxford Radcliffe (United Kingdom) and Karolinska (Sweden) hospitals; UPP: Uppsala Hospital (Sweden); VDX: Veridex (The Netherlands). These datasets were generated with diverse microarray technologies developed either by Agilent (www.genomics.agilent.com), Affymetrix (HGU GeneChips, which include chips HG-U133A, HG-U133B, and HG-U133PLUS2, and X3P GeneChip; www.affymetrix.com); Arcturus (http://products.invitrogen.com); Swegene (www.genomics.agilent.com), Operon (www.operon.com), or developed in-house (cDNA, cDNA, platforms). For most datasets survival data [distant metastasis-free survival (DMFS), relapse-free survival (RFS), and overall survival (OS)], the complete pathological response (pCR) and information regarding the adjuvant treatment (untreated, chemo, hormonal, and heterogeneous, standing for no treatment, chemotherapy, hormonal therapy, and heterogeneous combination of therapies, respectively) was available, otherwise missing information is referred to as not available (NA). All untreated patients had surgery, and most of them had radiation therapy, although information is not available for all datasets.

†Duplicated patients were removed from the UNT, UPP, MDA4, DFHCC2, DFHCC3, and TAM datasets for the estimation of concordance and prognostic value.

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Fig. S1. Triple-negative breast cancer (TNBC) as defined by the subtype classifier model (SCM) has similar levels of estrogen receptor (ESR1), progesterone receptor (PgR), and ERBB2 as the prediction analysis of microarray 50-gene classifier (PAM50)-defined "basal-like" subtype. ESR1, PgR, and ERBB2 expression values were rescaled within each dataset (see *Materials and Methods*). HER2, human epidermal growth factor receptor 2.

Fig. S1

Fig. S2. Boxplots showing CD73 gene expression is highest in TNBC subtype. CD73 expression values were rescaled within each dataset (see *Materials and Methods*). Kruskal–Wallis *P* value is shown (n = 6,209).

Fig. S2

Fig. S3. Gene expression profiles of 44 publically available microarray datasets were collected. Patients were assigned to the three main molecular subtypes using the SCM. Correlation (Spearman ρ) heatmaps of CD73 expression with ESR1 (single gene and gene module) and PLAU (plasminogen activator urokinase, invasion gene and gene module) are shown according to subtypes. Red indicates positive correlation, with green indicating inverse correlation and black indicating no correlation.

Fig. S3

Fig. S4. (*A*) Effect of CD73 overexpression in AT-3 mouse breast tumor cells (gray: unstained; doted: AT3-GFP; full line: AT3-CD73 cells). AT3-GFP and AT3-CD73 cells were treated with increasing doses of DOX for 48 h and cell viability was measured by colorimetric assay. (*B*) Effect of CD73 gene silencing by shRNA in MDA-MB-231 cells (gray: unstained; white: MDA-MB-231 shGFP; black: MDA-MB-231 shCD73 cells). MDA-MB-231 shGFP and MDA-MB-231 shCD73 cells were treated with increasing doses of DOX for 48 h and cell viability was measured by colorimetric assay. (*C*) 4T1.2 cells were treated with increasing doses of DOX with or without α,β-methyleneadenosine 5′-diphosphate (APCP; 100 μM) for 48 h and cell viability was measured by colorimetric assay. Means ± SEs of triplicates are shown. (*D*) Relative mRNA levels of Cd73, Bcl-2, p-glycoprotein and 18S in MDA-MB-231 shRNA cells compared with MDA-MB-231 shGFP cells (relative to 18S). (*E*) Subtypes and baseline CD73 expression levels in human breast cancer cells.

Fig. S4

Fig. S5. DOX treatment up-regulates CD73 expression in vivo. NOD-SCID mice were injected subcutaneously with MDA-MB-231 human breast tumor cells (10^6 cells) and treated when tumors reached 50 mm^2 (day 20) with an intratumoral injection of DOX (1 mM in 50 μL PBS) or PBS (control, CTR). Tumors were removed 48 h later for CD73 expression analysis. (*A*) IHC analysis of CD73 expression (in brown) in control-treated and DOX-treated tumors (20× magnification). (*B*) Whole tumor protein extracts were analyzed for CD73 and β-actin expression levels by immunoblotting ($n = 2$/group). (*C*) Same as *B*, except that a Li-Cor imager was used to measure CD73 expression.

Fig. S5

Fig. S6. Chemotherapy-induced CD73/CD39 up-regulation in breast cancer cells. Human breast cancer cell lines (i.e., MDA-MB-231, MDA-MB-468, SKBR3, BT474, ZR75, and T47D) were treated with increasing doses of DOX, cyclophosphamide (cyclophos), paclitaxel (PAC), 5-fluorouracil (5-FU), cisplatin, or oxaliplatin. CD73 and CD39 expression levels were measured by flow cytometry 48 h after treatment and reported as fold increase relative to untreated cells (means ± SEs of triplicates are shown).

Fig. S6

Fig. S7. Chemotherapy-induced CD73/CD39 up-regulation in melanoma and leukemia cells. Human melanoma (LOX-1MV1 and A2058) and leukemia (Kasumi-1 and RPMI-8226) cells were treated with increasing doses of DOX, cyclophosphamide (cyclophos), and 5- FU. CD73 and CD39 expression levels were measured by flow cytometry 48 h after treatment and reported as fold increase relative to untreated cells.

Fig. S7

Fig. S8. (*A*) Linear regression analysis of CD73 and CD39 up-regulation in response to chemotherapy (using data from Figs. S6 and S7 for DOX). (*B*) Linear regression analysis of CD73 (*Left*) and CD39 (*Right*) maximum up-regulation in human breast cancer cells (from Fig. S6) in response to DOX in relation to baseline CD73 and CD39 expression levels, respectively.

Fig. S8