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Amplification of LAPTM4B and YWHAZ contributes to chemotherapy resistance and recurrence of breast cancer

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Abstract

Post-surgery adjuvant chemotherapy for breast cancer has effectively reduced metastatic recurrence rates1. However, a significant proportion of women suffer recurrent cancer at distant metastatic sites despite adjuvant treatment. Identification of the genes critical for tumor response to specific chemotherapy drugs is a challenge, but necessary to improve outcomes2. Using integrated genomics, we identified a small number of over-expressed and amplified genes from chromosome 8q22 significantly associated with early disease recurrence despite anthracycline-

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AUTHOR CONTRIBUTIONS

A.L.R. and Z.C.W. designed the experiment and supervised the project. Y.L. (Yang Li) performed the *in vitro* laboratory experiments including generating cDNA vector constructs, gene transfer and knockdown, RT-PCR and Western blot analysis, and drug sensitivity and localization studies. L.Z. performed the PAM data analysis and statistics. Q.L., under supervision of Z.S., performed bioinformatic analysis on validation data sets and the cisplatin trial data set. R.T. performed apoptosis assays. Y.L. (Yan Li) contributed to the preparation of cDNA vector constructs. C.D., C.S., and B.H.-K. performed the epirubicin trial and provided clinical data and analysis. A.L.R. provided the DFHCC and cisplatin trial clinical samples and performed gene expression array analysis. Z.C.W. performed the SNP array analysis and scored the FISH assays. A.L.R., J.D.I. and Z.C.W. wrote the manuscript with comments from Y.L.(Yang Li), C.D., C.S., and Z.S.

based adjuvant chemotherapy. The association was confirmed in an analysis of multiple independent cohorts. Two of these genes, the anti-apoptotic gene *YWHAZ*, and *LAPTM4B*, a novel lysosomal gene, sensitized tumor cells to anthracyclines when either was depleted by siRNA knockdown and induced drug resistance when either was over-expressed. Over-expression of *LAPTM4B* resulted in sequestration of drug, delaying its appearance in the nucleus. Over-expression of these two genes was associated with poor tumor response to anthracycline treatment in a neo-adjuvant chemotherapy trial in women with primary breast cancer. Our results suggest that 8q22 amplification and over-expression of *LAPTM4B* and *YWHAZ* contribute to *de novo* chemoresistance to anthracyclines, and are permissive for metastatic recurrence. These two genes may predict anthracycline resistance and influence selection of chemotherapy.

Breast cancer recurs at distant sites in a significant number of women who receive adjuvant chemotherapy after surgical removal of the primary breast tumor1. *De novo* resistance mechanisms present within tumor cells prior to treatment are key factors leading to failure of chemotherapeutic drugs to prevent metastatic recurrence. Therefore, discovery of the genomic alterations and genes contributing to *de novo* chemo-resistance to specific drugs is an important goal2. Although a number of multidrug resistance genes have been discovered, their over-expression is often induced during drug treatment3,4 and not useful for initial guidance of drug selection. Gene signatures generated from drug responses of tumor cell lines are reported to predict drug response in patients5–7; however, others found cell-line derived signatures are not predictive of response in clinical cases8. Repeatedly observed genomic gains or losses have identified genomic regions that may harbor genes contributing to malignant behavior and poor outcome9–12. Which genomic region(s) harbors genes that may contribute to *de novo* resistance to therapy is currently unknown.

We analyzed gene expression profiles of 115 breast carcinomas from women diagnosed between 2000 and 2003 and treated according to current guidelines including adjuvant chemotherapy if indicated. We performed predictive analysis of microarrays (PAM; 13) and identified 114 probes, encoding 75 known genes, differentially expressed between cases with early distant metastatic recurrence and cases without distant recurrence (Supplementary Table 1). Fifteen percent of these probes, corresponding to 12 different genes, mapped to chromosome 8q22, the only chromosomal region with statistically significant enrichment ($P < 2.1e-09$) of probes associated with metastatic recurrence (Fig. 1a). We applied Cox proportional hazard regression 13,14 which also demonstrated differential over-expression of these 8q22 genes in tumors with distant recurrence. These genes included *CCNE2* and *MTDH* which are reported associated with metastatic recurrence and poor prognosis of breast cancer 15,16. The coordinate over-expression of neighboring genes often reflects chromosomal amplification. Indeed, 8q22 amplification was observed by SNP array analysis in 50 breast cancers (Supplementary Fig. 1) and expression of the 8q22 genes correlated with DNA copy number (Supplementary Table 1).

We confirmed 8q22 amplification by DNA interphase fluorescence in situ hybridization (FISH) (Fig. 1b,c) and found it in 21% of 85 breast cancers. Degree of copy gain was correlated with average expression of the 12 recurrence-associated 8q22 genes (8q gene expression index, 8qEI) (Fig. 1d and Supplementary Table 1). Kaplan-Meier analysis

showed 8q22 amplification was associated with reduced metastasis-free survival in the entire cohort evaluated by FISH (Fig. 1e), in the ER− cases (Supplementary Fig. 2a), and in the women who had received anthracycline-based adjuvant chemotherapy (Fig. 1f). In multivariate analysis, amplification of 8q22 was a strong independent prognostic factor for breast cancer recurrence (Supplementary Table 2).

We sought validation in a meta analysis of six independent cohorts annotated with treatment and outcome 12,14,17–20. Kaplan-Meier analysis demonstrated a significant difference in disease-free survival between 8qEI low-expression and high-expression groups in either chemo-treated (Fig. 1g) or untreated cases (Supplemental Fig.2b). These results indicate that 8q22 amplification promotes over-expression of 8q22 genes in tumor tissue, which are associated with poor prognosis in untreated cases and inferior disease-free survival despite adjuvant chemotherapy.

To determine if 8q22 genes influence sensitivity to chemotherapy, we treated the breast cancer cell line BT549 harboring 8q gain with siRNA against the 12 candidate genes (Supplementary Fig. 3) and screened for alteration of sensitivity to chemotherapeutic drugs (Fig. 2a). Depletion of two genes significantly increased the sensitivity to anthracyclines (Fig. 2a). One of these, *YWHAZ*, codes for a known anti-apoptotic protein 14-3-3ζ 21,22. The second gene codes a novel lysosomal protein LAPTM4B (Lysosomal Associated Protein Transmembrane 4B), about which little is known in breast cancer. We examined 16 breast cancer cell lines and found a strong positive correlation between higher endogenous *LAPTM4B* mRNA level and higher IC_{50} (relative resistance) to anthracyclines ($P < 0.00034$, Supplementary Fig. 4a), a weaker or no correlation with IC_{50} to cisplatin and paclitaxel ($P = 0.008$ and 0.4, respectively; data not shown). The expression of *YWHAZ* in cell lines also correlated with the IC_{50} to doxorubicin (Supplementary Fig 4). Specific knockdown of LAPTM4B and YWHAZ in three cell lines (Fig. 2b) increased sensitivity to the anthracyclines doxorubicin (Fig. 2c upper panels) and daunorubicin (data not shown), but had weaker or no effect on sensitivity to cisplatin and paclitaxel (Fig. 2c lower panels). Knockdown of either LAPTM4B or YWHAZ significantly increased doxorubicin-induced apoptosis (Fig. 2d). Induction of apoptosis was less apparent in response to cisplatin treatment (Fig. 2d).

To investigate mechanism, intracellular localization of anthracyclines was tracked by following the autofluorescence of doxorubicin. *LAPTM4B* expression in cell lines correlated with both IC_{50} of doxorubicin (Fig. 3a) and with appearance of doxorubicin in the nucleus within 24 hours (Fig. 3b). Knockdown of LAPTM4B by siRNA in MDA-MB-231 cells resulted in a significant increase in nuclear localization of doxorubicin, detectable within 12 h to 24 h of treatment, maximal at 24 h to 36 h (12 h after withdrawal of drug), and sustained at 48 h (Fig. 3c). Knockdown of LAPTM4B in BT549 cells resulted in a similar increase in nuclear localization at 24 h (data not shown). Decreased distribution of doxorubicin into the nucleus is associated with lower phospho-H2AX (Fig. 3c,d), a marker of DNA damage response, and consistent with reduced doxorubicin-induced apoptosis and increased IC_{50} (Fig. 2). YWHAZ levels correlated with IC_{50} of doxorubicin (Fig. 3a) and knockdown of YWHAZ increased slightly the phospho-H2AX levels in drug treated cells (Fig. 3d). These results suggest one mechanism of poorer outcome for women harboring

breast cancers with 8q22 amplification is increased expression of *LAPTM4B* and interference with nuclear accumulation of anthracyclines. YWHAZ may effect drug sensitivity through inhibition of apoptosis, consistent with reports of others21,22.

We introduced HA-tagged full length *LAPTM4B* and *YWHAZ* into partially transformed human mammary epithelial cells (HMECs) 23. Expression of either exogenous LAPTM4B or YWHAZ increased the IC_{50} of doxorubicin (270% increase, $P < 0.002$ and 394% increase, $P = 0.0001$, respectively) but had no significant effect on sensitivity to paclitaxel or cisplatin (Fig. 3e, f). The LAPTM4B-induced decrease in drug sensitivity parallels and is consistent with delayed appearance of anthracycline in the nucleus of LAPTM4B-overexpressing HMEC (Fig. 3g).

Kaplan Meier analysis of women treated with adjuvant chemotherapy showed that the expression of *YWHAZ* and *LAPTM4B* above median level was associated with shorter disease-free survival (Supplementary Fig. 2c). The association with poor outcome after adjuvant chemotherapy is consistent with either a prognostic effect or a role of these two genes in chemotherapy resistance.

Finally, *LAPTM4B* and *YWHAZ* were tested for their association with response to anthracyclines in a neoadjuvant (pre-operative) treatment trial of epirubicin monotherapy. The average of *LAPTM4B* and *YWHAZ* expression levels from expression array data of pretreatment tumor biopsies was evaluated for association with pathologic complete response (pCR) to epirubicin. The two-gene expression levels displayed a coherent pattern with higher levels of expression associated with absence of pCR (presence of residual disease) after epirubicin treatment (Fig. 4a, b). We evaluated the capability of the two genes to predict pCR by measuring the area under receiver operator characteristic (ROC) curves24,25(AUC); which demonstrated higher expression of the two genes is associated with absence of pCR after anthracycline chemotherapy in the cohort of 118 breast tumors (AUC 0.315, $p < 0.00058$, Fig. 4d). The association is more significant in 87 ER^- $HER2^-$ tumors (AUC 0.241, $p < 0.000062$, Fig. 4e). When the two genes were analyzed separately, both genes were significantly predictive of poor response in the whole cohort and in the ER^- $HER2^-$ subset, but only LAPTM4B level was predictive in the ER^- $HER2^+$ subset (Supplementary Table 3). In contrast, the expression of these two genes was not associated with treatment response to cisplatin monotherapy in a separate neo-adjuvant clinical trial in triple negative (ER^- PR^- $HER2^-$) cancer cases26 (AUC 0.675, $P > 0.3$, Fig. 4c,f; Supplementary Table 3). Although *MTDH*, one of the twelve 8q22 genes, was reported to induce chemo-resistance to a broad spectrum of drugs in experimental models16, its expression was not predictive for pCR in either the epirubicin or cisplatin trials (data not shown). We analyzed a third single agent neoadjuvant therapy trial of predominantly ER^+ tumors treated with docetaxel27 and found that higher levels of expression of *LAPTM4B* and *YWHAZ* were not associated with an inferior clinical response to therapy (data not shown). The results support the notion that *LAPTM4B* and *YWHAZ* over-expression is associated preferentially with poor response to anthracyclines.

LAPTM4B is similar to its family member LAPTM4A that promotes selective resistance to anthracyclines and not cisplatin in Saccharomyces cerevisiae 28. Our results show that

LAPTM4B acts on anthracycline trafficking by reducing drug entry into the nucleus and decreasing drug-induced DNA damage. Higher *YWHAZ* expression protects cells from drug-induced apoptosis. Because they reside in proximity, amplification produces coordinated up-regulation of their various functions, together resulting in preferential resistance to anthracyclines. The results from three clinical trials support this contention, and suggest clinical options for the treatment of primary breast cancers might depend upon the status of 8q22 amplification and over-expression of these two genes in tumors. Anthracyclines appear to be reasonable treatment in tumors without 8q22 amplification, and alternatives might be selected for those whose cancers harbor amplification.

Microarray data

Microarray data sets are deposited in the NCBI GEO database under the following accession numbers: Gene expression data from the DF/HCC cases, GSE19615; SNP array data from 50 of the DF/HCC cases, GSE19594; gene expression data from neo-adjuvant epirubicin "Trial of Principle", GSE16446; gene expression data from neo-adjuvant cisplatin trial, GSE18864.

Methods

Cohort

Primary breast tumors of 115 subjects were obtained from the NCI-Harvard Breast SPORE blood and tissue repository under Dana-Farber/Harvard Cancer Center Institutional Review Board approved protocols, with informed consent from subjects. Affymetrix U133 plus 2 gene expression array analysis was performed as described 29,30 A subset of 85 tumors were represented in tissue microarrays and used for FISH analysis (see Supplementary Methods). Fifty of the cases were analyzed by Affymetrix 10K SNP array as described31,32. A portion of the SNP and gene expression data were reported previously 29,30,31,32. Clinical and pathologic characteristics for each sample in the cohort are provided in Supplementary Table 4.

Neoadjuvant Clinical Trials

The neoadjuvant "Trial of Principle" for breast cancer is conducted in European hospitals and coordinated at the Institut Jules Bordet. This trial is registered on the clinical trials site of the US National Cancer Institute website http://clinicaltrials.gov/ct2/show/NCT00162812?term=NCT00162812&rank=1. Single agent epirubicin was given as neo-adjuvant (pre-operative) chemotherapy to 118 reportedly ER^- cases. After central review, 4 of the cases were found to be ER^+. Of the remaining 114 ER^-, 87 cases were classified as $HER2^-$ based on low ERBB2 module score33. Pretreatment core biopsy of the primary breast tumor was performed for diagnosis and RNA isolation. At completion of chemotherapy, pathologic response was determined by microscopic examination of the excised tumor and nodes. Pathological complete response (pCR) was defined by the absence of residual invasive breast carcinoma in the breast and axillary nodes. This study has been approved by the medical ethics committee of Institute Jules Bordet and all women given

written informed consent prior to study entry. Gene expression data of U133plus 2 were generated from RNA of pretreatment core biopsies.

The trial of single agent cisplatin given as neo-adjuvant chemotherapy to women with triple negative breast cancer26. Gene expression array data from pre-treatment biopsies was available for 24 cases26. Pathological response was determined by microscopy examination after chemotherapy as described above.

ROC (Receiver Operating Characteristic) curve analysis was performed to evaluate the mean level of combined *LAPTM4B* and *YWHAZ* expression, or the levels of each individual gene, for their capacity to predict pathological complete response (pCR). The association of ranked gene expression levels with pCR was evaluated by determining the area under the curve (AUC) estimated through the concordance index34; the corresponding p-value is from one-sided Wilcoxon's rank test35. The ROC curves are plotted for prediction of pCR, so a curve below the midline in low-right area of the graph indicates the ranked gene expression is associated with absence of pCR.

Statistical analysis of gene expression arrays

Affymetrix U133plus2.0 array data from tumor samples of 115 cases were classified into those with distant recurrence within 36 months of diagnosis or those without distant recurrence and at least 36 months of follow up. The 115 arrays were log-transformed, normalized and invariable genes were removed by filtering using dChip software (p-value < 0.05). Differentially expressed genes were determined by Prediction Analysis of Microarrays (PAM) 13 using the *pamr* package implemented in R language (http://cran.r-project.org/web/packages/pamr/index.html). PAMR implements the shrunken nearest centroid method 13. Genes were selected at a false discovery threshold that minimized a 10-fold cross-validation and test errors near the shrinkage parameter $\Delta = 2$. The PAM score indicates the degree of statistical association for expression of each gene and metastatic recurrence. Cox proportional hazard regression analysis 13,14 was used to discover differentially expressed genes associated with time-to-recurrence.

Analysis in independent cohorts

Pooling six independent gene expression array datasets 12,14,17–20 for analysis was performed as described 36. 8q22 gene expression-based Index (8qEI) is calculated as the mean expression value of the twelve 8q22 genes identified in PAM analysis, and was scored in each of the six independent cohorts. We defined a simple median-based classifier: 8qEI level higher than the median as 8qEI high and lower than the median as 8qEI low. The classification was performed in 6 cohorts separately, and then combined into one data sheet with 1348 samples. Of these, 1130 had annotation for treatment and followup, 361 cases received adjuvant chemotherapy with or without hormonal therapy and 725 cases received no adjuvant hormonal or chemotherapy. Follow-up time was constrained to a maximum of 10 years with annotation for disease free survival. Kaplan-Meier analyses were carried out using the survival package within the R statistical package. *P*-values are derived using Mantel-Cox logrank test.

Additional methods are provided in supplementary material online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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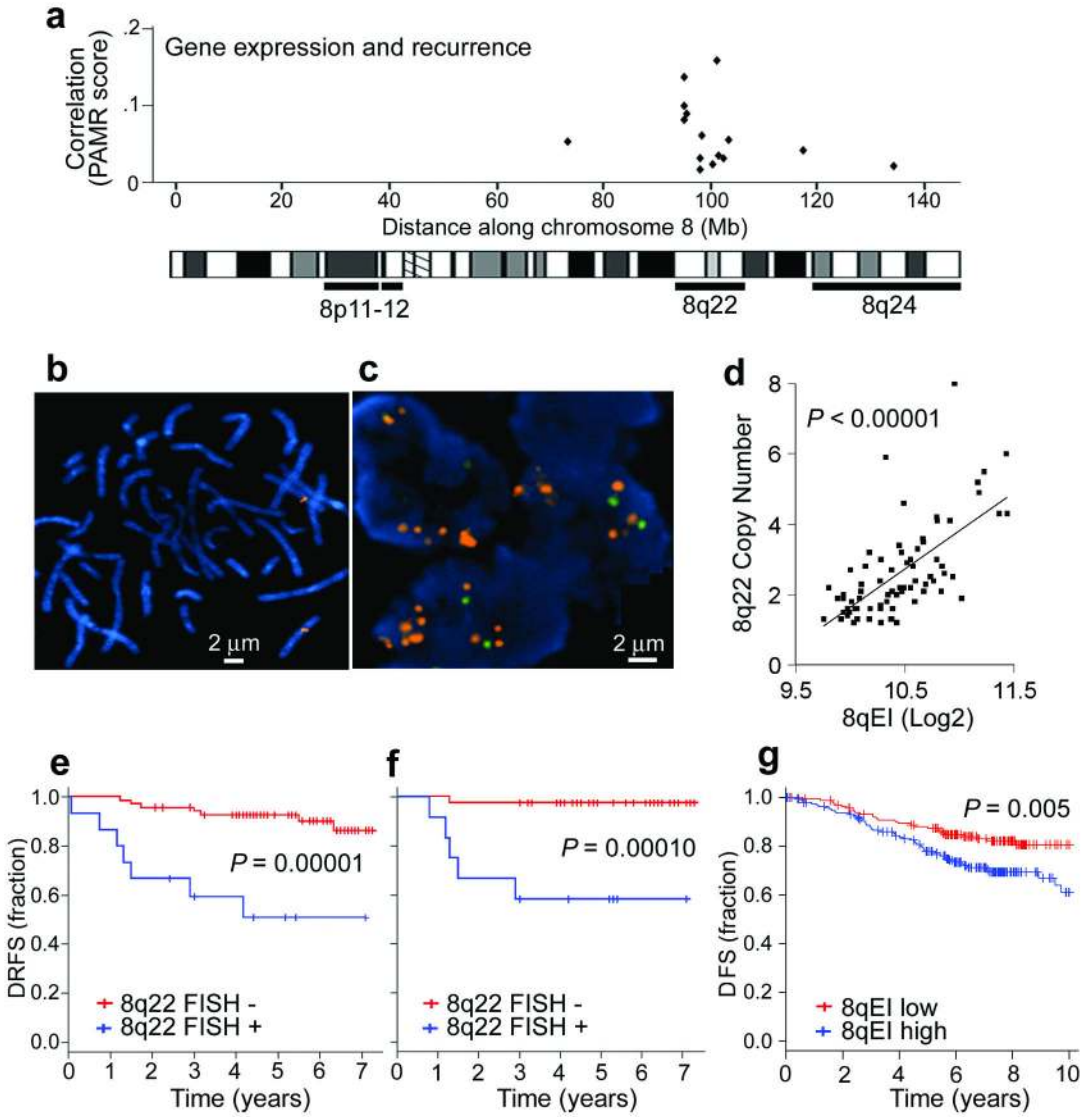

Figure 1. 8q22 amplification, gene expression and cancer recurrence

(**a**) Values on the ordinate are the PAM score. Genes with significant PAM scores are displayed according to their annotated location on chromosome 8 (**b**) metaphase FISH in normal human lymphoid cells using 8q22 probe RP11-347C18 (orange) (**c**) interphase FISH with 8q22 probe RP11-347C18 (orange), chromosome 8 centromere probe (green), and DAPI nuclear stain (blue) in a human tumor sample (**d**) Correlation between 8q22 copy number detected by FISH and the 8q22 twelve gene expression-based index (8qEI) (regression R = 0.65). (**e**) Kaplan-Meier analysis for distant recurrence-free survival (DRFS) of 85 Boston cases with or without 8q22 amplification identified by FISH (Cox Hazard Ratio (HR) = 7.77). (**f**) DRFS for 52 Boston cases treated with doxorubicin and cyclophosphamide, according to 8q22 amplification identified by FISH (HR = 7.66). (**g**) Disease free survival (DFS) for 361 patients from six independent cohorts who received adjuvant chemotherapy, according to 8qEI high and low based on 8qEI levels above or below median level in each of the six cohorts (HR = 1.9).

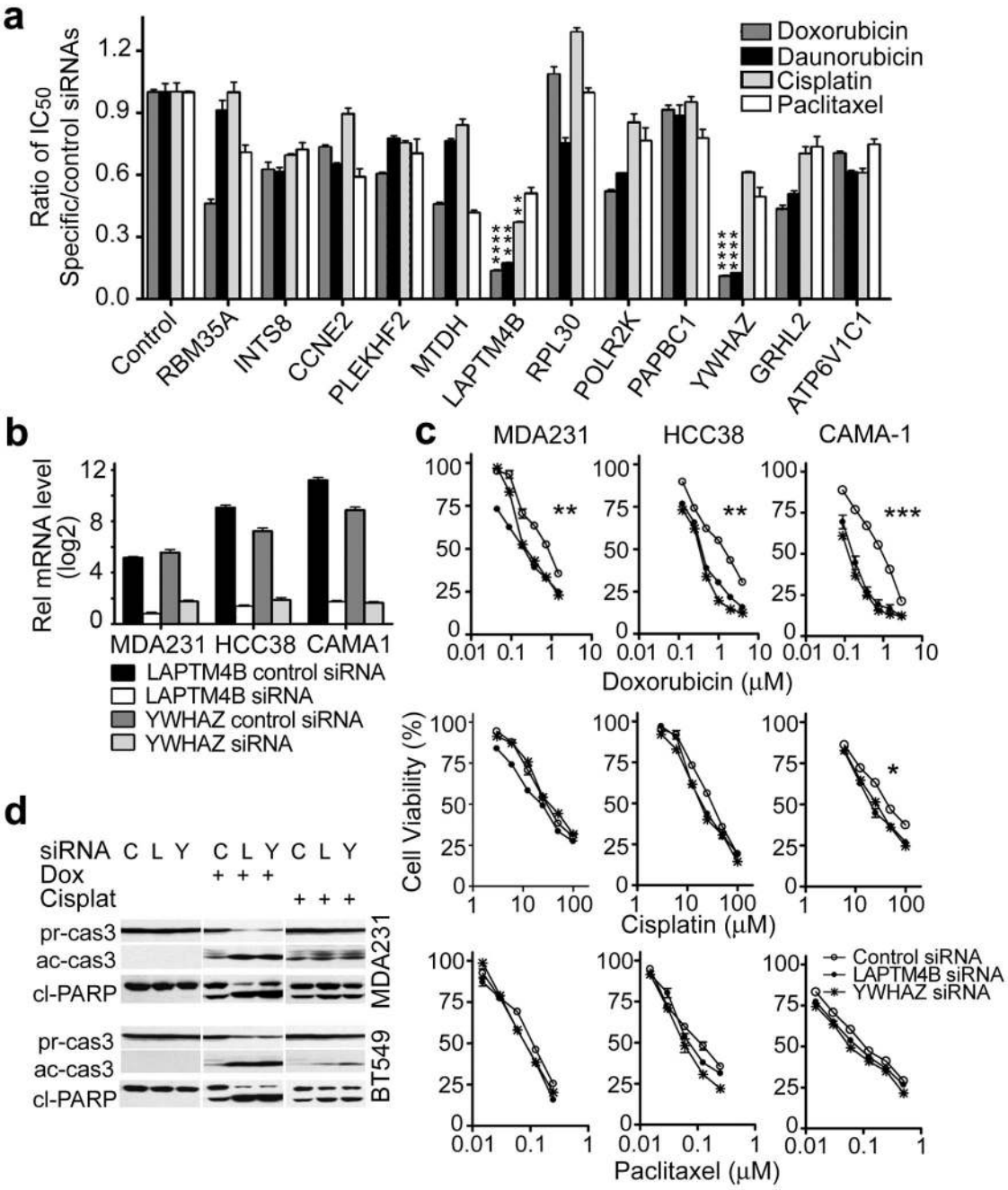

Figure 2. Knockdown of 8q22 genes by siRNA in tumor cell lines to determine effect on sensitivity to anthracycline chemotherapy

(**a**) siRNA knockdown of the twelve 8q22 genes in BT549 breast tumor cells. Bars indicate the ratio of IC_{50} for the indicated drugs in gene-specific siRNA treated cells, relative to control siRNA treated cells. Percent reduction in IC_{50} is indicated as follows: ** 63% reduction, *** 82%, **** >85%. (**b**) Relative mRNA levels for LAPTM4B and YWHAZ in the three breast cancer cell lines, after treatment with control or specific siRNAs as indicated. Levels are log 2 scale and relative to HMEC reference sample. (**c**) Drug

concentration-dependent cell survival curves for doxorubicin (upper panels), cisplatin (middle panels), and paclitaxel (lower panels) in cells transfected with control siRNA or gene-specific siRNAs, as indicated. The cell lines are shown at top. Approximate percent reduction in IC50 is indicated as follows: *** 85%, ** 75%, and * 50%. (**d**) Western blot for pro-caspase 3 (pr-cas3), active caspase 3 (ac-cas) and cleaved lower molecular weight PARP (cl-PARP), in MB231 and BT549 cells transfected with siRNA for LAPTM4B (L), YWHAZ (Y), or scramble control (C) as indicated across the top. Cells were treated with carrier (no marker) or with doxorubicin or cisplatin (+) for 48 hours, as indicated above each lane.

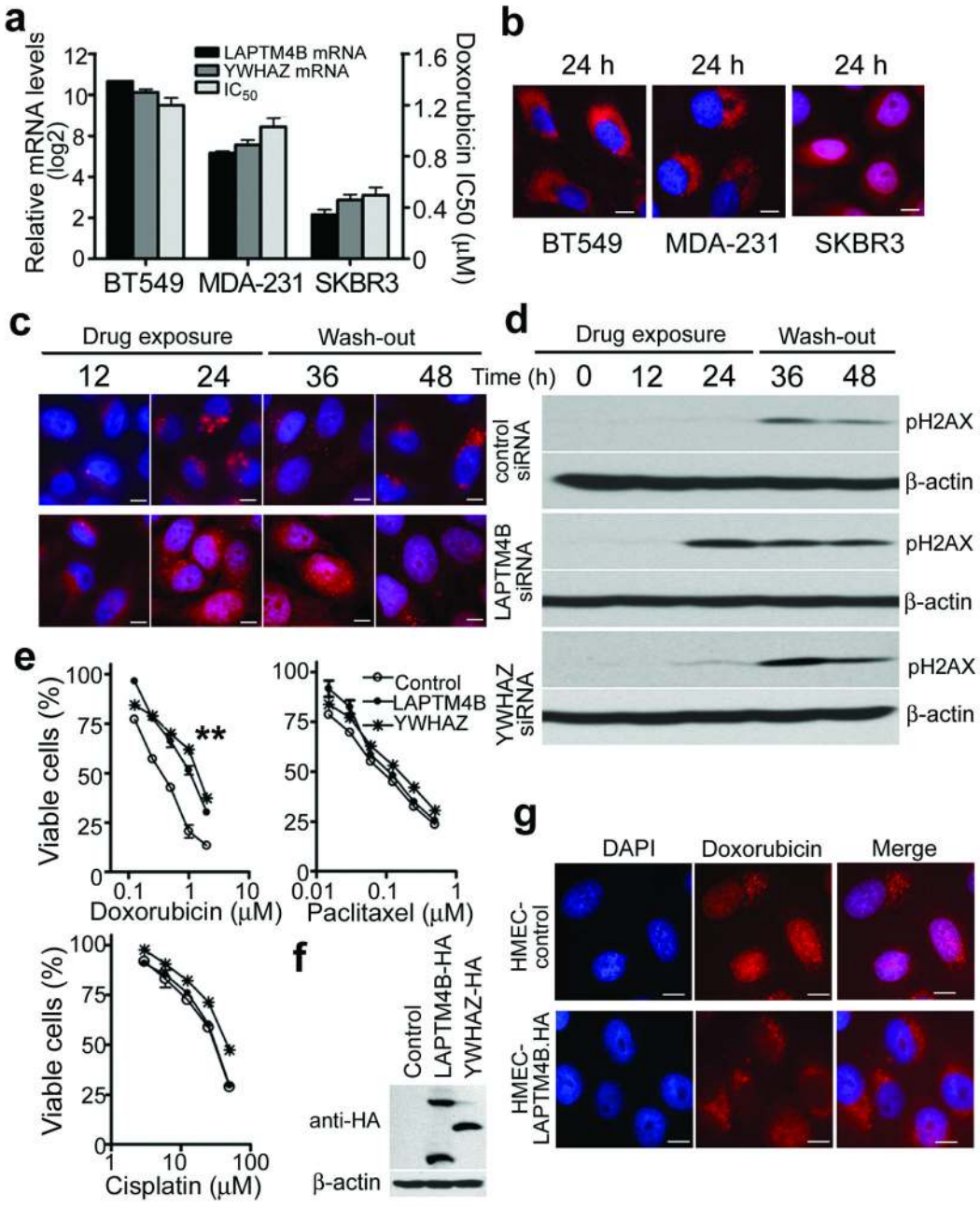

Figure 3. *LAPTM4B* expression, intracellular doxorubicin distribution, and drug-induced DNA damage in breast cancer cell lines

(**a**) mRNA levels of LAPTM4B and YWHAZ relative to a reference sample (log2 scale, left axis) and doxorubicin IC_{50} concentration (μM, right axis) in three breast cancer cell lines, as indicated below the bars. (**b, c, and g**) Merged fluorescence analysis for doxorubicin (drug autofluorescence, red) and nuclear staining (DAPI, blue). Doxorubicin localized in the nucleus appears purple. Scale bars indicate 10 μm. (**b**) Intracellular doxorubicin distribution after 24 h of drug exposure in three breast tumor cell lines. (**c**) Intracellular doxorubicin

distribution in MDA-MB-231 cells transfected with control siRNA and LAPTM4B-specific siRNA as indicated to the right. Time points during drug exposure and after removal of drug from culture medium are shown above the panels. (**d**) Western blot with anti-phospho-H2AX antibody and β-actin control antibody in lysates of MDA-MB-231 cells transfected with control and gene-specific siRNA oligonucleotides, as indicated along the left side, at the indicated time points of drug exposure and after removal of drug from culture medium. (**e**) Plot of inhibition of cell growth by doxorubicin, paclitaxel and cisplatin in HMEC cells transfected with vectors containing GFP control, *LAPTM4B*-HA, or *YWHAZ*-HA. The percent viable cells, compared to transfected cells without drug treatment, are indicated on the Y-axis (mean of triplicates ± S.D.). ** indicates >250% increase in IC_{50}. (**f**) Western blot of HMEC cells transfected with vectors containing LacZ control, *LAPTM4B*-HA and *YWHAZ*-HA, as indicated above, using HA tag antibody and β-actin antibody. (**g**) Intracellular doxorubicin distribution in HMEC cells transfected with LacZ control vector or with *LAPTM4B*-HA vector after 16 hours of doxorubicin exposure.

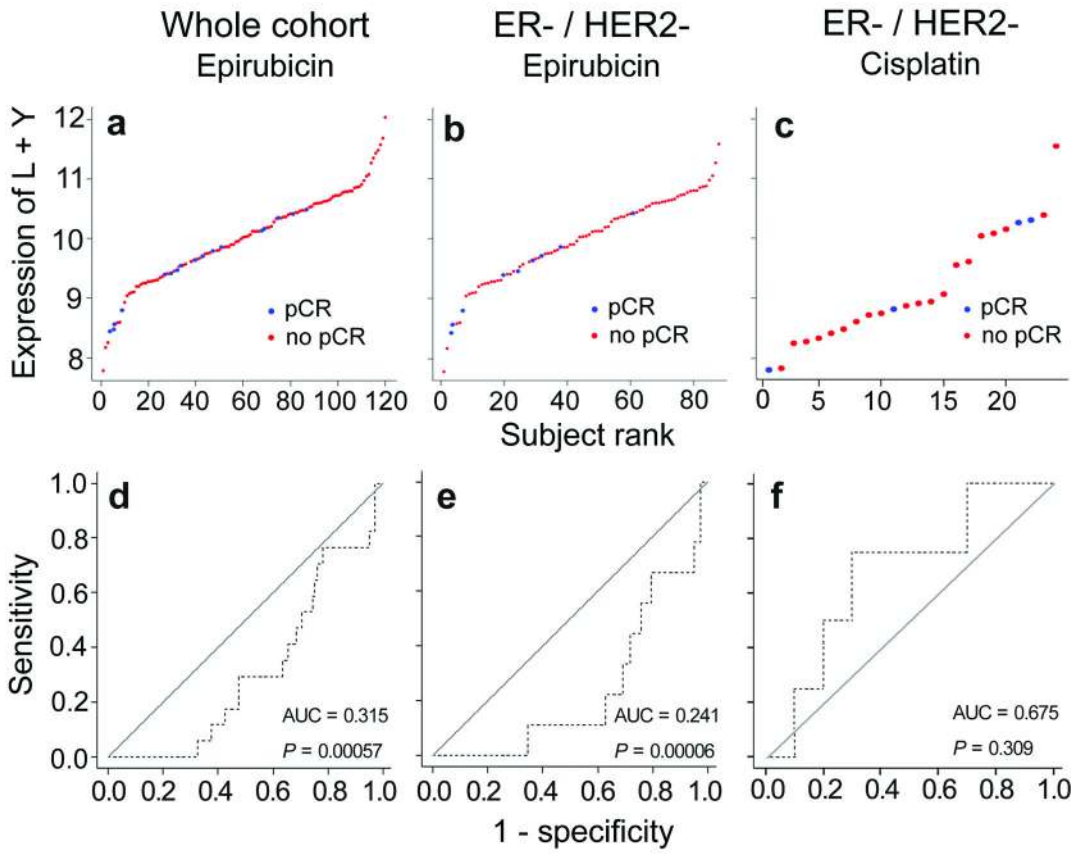

Figure 4. ***LAPTM4B*** **and** ***YWHAZ*** **expression and pathologic complete response (pCR) to neoadjuvant chemotherapy**

(**a, b, c**) Cases ranked according to mean sum expression of LAPTM4B and YWHAZ and (**d, e, f**) Receiver Operating Characteristic plots for the performance of the LAPTM4B and YWHAZ genes to predict pCR for (**a, d**) epirubicin in the whole breast cancer cohort (pCR, $n = 17$; no pCR, $n = 101$), (**b, e**) epirubicin in the ER$^-$ and HER2$^-$ sub-cohort (pCR, $n = 9$; no pCR, $n = 78$), and (**c, f**) cisplatin in ER$^-$ PR$^-$ HER2$^-$ breast cancer patients (pCR, $n = 4$; no pCR, $n = 20$). In the ROC plots (**e, f, g**), the solid diagonal lines indicate the performance of a random predictor and the dashed line indicates the performance of the mean LAPTM4B and YWHAZ levels to predict pCR.