Protein phosphatase 1, regulatory subunit 15B is a survival factor for ERα-positive breast cancer

Maria Shahmoradgoli1, Yasser Riazalhosseini2*, Daniel Haag1, Natalia Becker1, Volker Hovestadt1, Stefanie Heck1, Hans-Peter Sinn3, Andreas Schneeweiss4, Otto Mannherz1, Özgür Sahin5 and Peter Lichter1

1 Division of Molecular Genetics, German Cancer Research Center, Heidelberg, Germany
2 Division of Functional Genome Analysis, German Cancer Research Center, Heidelberg, Germany
3 Institute of Department Gynecological Pathology, University of Heidelberg, Heidelberg, Germany
4 National Center for Tumor Department Gynecological Oncology, University Hospital, University of Heidelberg, Heidelberg, Germany
5 Division of Molecular Genome Analysis, German Cancer Research Center, Heidelberg, Germany

Breast cancer is a heterogeneous disease at both the clinical and molecular levels. This heterogeneity may give rise to different therapy responses. Molecular profiling has facilitated identification of signatures for stratifying patients who would potentially benefit from given therapies. Previously, we reported on a subset of genes with the potential for predicting response of primary breast cancer to neoadjuvant chemotherapy. Herein, we report that patients with luminal (estrogen receptor α [ERα]-expressing) breast cancer were enriched for nonresponders. To identify novel factors that contribute to the survival of breast cancer cells, a loss-of-function screen was performed with a subset of genes overexpressed in patients with disease resistant to chemotherapy. This approach led us to identify protein phosphatase 1, regulatory subunit 15B (PPP1R15B) as a factor with a potentially essential role in the survival of ERα-positive breast cancer cells. Functional analyses showed that PPP1R15B depletion results in impaired proliferation due to unsuccessful transition of cells from G1 to S phase of the cell cycle, and apoptosis induction. Moreover, our data revealed a regulatory role for PPP1R15B in activating ERα.

Furthermore, a high level of PPP1R15B mRNA expression was associated with poor outcome following tamoxifen-based therapy. Accordingly, knockdown of PPP1R15B expression sensitized tamoxifen-resistant MCF-7 breast cancer cells to tamoxifen while reducing ERα abundance in these cells. Our findings reveal a novel role for PPP1R15B in the survival and therapy response of ERα-positive breast cancer and may open new avenues for tumor subtype-specific therapeutic strategies in the era of personalized medicine.

Breast cancer is composed of molecularly distinct subtypes, and drug resistance has been attributed to molecular signatures as well. Therefore, current efforts focus on the identification of patients who most likely respond to certain treatments and the development of novel therapeutic strategies for patients predicted to be nonresponders.1–4

Key words: PPP1R15B, breast cancer, estrogen receptor α, tamoxifen, drug resistance, cell proliferation

Abbreviations: ERα, estrogen receptor α; non-pCR, nonresponders; pCR, pathological complete remission; pPR, pathological partial remission; pNC, pathological no change; PPP1R15B, protein phosphatase 1, regulatory subunit 15B; RNAi, RNA interference

Additional Supporting Information may be found in the online version of this article.

*M.S. and Y.R. contributed equally to this work

Grant sponsor: German Cancer Research Center (DKFZ)

DOI: 10.1002/ijc.27945

History: Received 26 Sep 2012; Accepted 19 Oct 2012; Online 20 Nov 2012

Correspondence to: Peter Lichter, Division of Molecular Genetics, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 580, 69120 Heidelberg, Germany, Tel.: +49-6221-42-4639, Fax: +49-6221-42-4639, E-mail: Peter.lichter@dkfz.de

Molecular profiles have also been used to identify novel pathogenic factors and pathways critical to the fitness of malignant cells. Of the genes overexpressed in mammary carcinomas that do not respond to therapy, some presumably have oncogenic functions, whereas of the downregulated genes in the same tumors, some are likely endowed with tumor suppressor activities. Thus, both loss- and gain-of-function screens of such gene populations will identify genes potentially involved in cellular processes implicated in therapy resistance of cancer cells.

Studies have shown that dysfunction of apoptotic pathways and uncontrolled cell proliferation can contribute to unsuccessful therapy.5,6 However, not all the factors involved in deregulation of these processes are known. In this study, we conducted an in vitro RNA interference (RNAi)-based loss-of-function screen of subset of genes overexpressed in cancer cells in patients with nonresponding breast cancer aimed at the identification of survival factors.

Materials and Methods

Statistical

The PAM50 classifier7 was applied to gene expression profiles from the microarray dataset to assign patient samples to the intrinsic subtypes. Statistical significance was determined
using the Student’s *t*-test on normally distributed data. *p* values <0.05 were considered statistically significant. Data are presented as mean values ± SD from three independent experiments, unless otherwise noted.

**Cell culture, transfections and measurements of cell viability and activated caspases**

MCF-7, MDA-MB-231, BT-474, EVSAT, T47D and SK-BR-3 cells were cultured under standard conditions. Cell line authentication was assessed using short tandem repeat profiling. Cells (5 × 10^5 cells/well in 96-well culture plates) were transiently transfected with individual siRNAs (50 nM) using the siPORT NeoFX transfection agent (Ambion, Austin, Texas www.ambion.com). Seventy-two hours later, cell viability was measured using a CellTiter Glo assay (Promega, Karlsruhe, Germany www.promega.com) according to the manufacturer’s instructions. Activation of caspase 3/7 was determined 48 hr after transfection of cells with siRNAs using the Caspase-Glo 3/7 assay kit (Promega).

**Cell cycle analysis and proliferation assay**

Cells were plated in 24-well culture plates at a density of 4 × 10^4 cells/well and transfected with 50 nM siRNAs. After 48 hr, cells were harvested and stained for cell cycle analysis using propidium iodide. The DNA content was analyzed using a FACScanto II flow cytometer (BD Bioscience, San Jose, USA www.bdbiosciences.com). The rate of DNA synthesis was evaluated by Click-iT 5-ethynyl-2'-deoxyuridine (EdU) flow cytometry assay kit (Invitrogen, Karlsruhe, Germany www.invitrogen.com). Forty-eight hours after siRNA transfection, cells were incubated for 16 hr at 37°C in presence of 10 μM EdU. Cells were then harvested, permeabilized and detected using a FACScanto II flow cytometer according to the manufacturer’s instructions. Ten thousand cells were measured for flow cytometric analysis of each sample.

**Western blotting**

Protein lysate preparation and Western blot analysis were performed as described previously. Antibodies: protein phosphatase 1, regulatory subunit 15B (PPP1R15B), Glyceraldehyde 3-phosphate dehydrogenase and Tubulin (Sigma-Aldrich, Munich, Germany www.sigmaaldrich.com), Cyclin D1 and phospho-Rb (Cell Signaling Inc, Danvers, USA www.cellsignal.com) and Beta-actin (MP Biomedicals, Eschwege, Germany www.mpbio.com).

**Luciferase reporter assay**

Reporter assays were performed as previously described.

**Drug sensitivity assay**

Survival of tamoxifen-resistant cells was examined upon siRNAs transfection under 4-hydroxytamoxifen at different concentrations or ethanol solvent control. Forty-eight hours thereafter, cell viability was measured using the Cell Titer Glo assay.

**Results and Discussion**

**Genes highly expressed in breast cancer patients not responding to chemotherapy**

Recently, we exploited the potential of a gene expression signature to predict the response of breast cancer patients to the neoadjuvant chemotherapy. According to clinical behavior, patients were classified as responders only in case of pathological complete remission (pCR) after primary systemic chemotherapy. We designated patients with residual tumor cells at surgery resulting in either pathological partial remission or pathological no change (pNC) as nonresponders (non-pCR).

Because genes highly expressed in breast cancer samples obtained from patients with pNC but not in pCR may encode cell death inhibitors or cell survival factors leading to therapy resistance, we performed significance analysis of microarrays for a series of published gene expression data of nine pCR versus six pNC breast tumor samples. Of 345 differentially expressed genes, 96 were highly expressed in the pNC group (Supporting Information Table S1) and thus were considered candidates for further analysis. When these gene expression data were used to classify patients according to the intrinsic breast cancer subtypes, we observed that the proportion of nonresponders was particularly high in patients with luminal A and B tumors (Fig. 1). As these two subtypes are commonly positive for expression of hormone receptors, the presence of estrogen receptor α (ERα) was significantly associated with the state of non-pCR (*p* < 0.001). These findings were supported by an analysis of another publicly available gene expression dataset of breast cancer patients obtained prior to neoadjuvant chemotherapy. This indicates that enhanced ER signaling is involved in at least supportive for cellular mechanisms of evasion of chemotherapeutic intoxication. Therefore, the subsequent loss-of-function screen of candidate genes was conducted in ERα-positive breast cancer cells.
Identification of PPP1R15B as a crucial factor for MCF-7 cell viability by small interfering RNA screen

To identify genes that induce reduction in cell viability and/or proliferation upon silencing, we performed an in vitro loss-of-function screen of 69 of the 96 genes (based on small interfering RNA [siRNA] availability) highly expressed in the pNC group. ERα-positive MCF-7 cells were transiently transfected with individual siRNAs targeting the respective genes and cell viability was measured 72 hr later. The targets of those siRNAs that decreased the level of cell viability by 50% (relative to that of the negative control) were considered to potentially contribute to cell survival. Following this screen, we identified PPP1R15B as the candidate gene with the strongest impact on the viability of MCF-7 cells (Fig. 2a, 2b, 2c, 2d).

Figure 1. High proportion of nonresponders to neoadjuvant chemotherapy in luminal subtypes of breast cancer. Distribution of pCR or non-pCR across intrinsic breast cancer subtypes in two different datasets (p < 0.001 and p = 0.004, Fisher’s exact test).

Figure 2. Identification of PPP1R15B as a survival factor in breast cancer cells. (a) Upon screening with siRNAs against 69 genes upregulated in patients with pNC to assess their effect on cell viability, PPP1R15B was identified as the top hit. The results were normalized to the viability of negative control, which was set to 1.0. The dashed line shows the value corresponding to the top candidate PPP1R15B relative to the negative control. (b) Measurement of the viability of MCF-7 cells transfected with three PPP1R15B siRNAs targeting different regions of the PPP1R15B mRNA (*p < 0.05) (siRNA information are presented in supplemental data). (c) Activation of caspase-3/7 was measured in MCF-7 cells transfected with si-PPP1R15B (**p < 0.03). (d) Western blot analysis of PPP1R15B protein in MCF-7 cells transfected with PPP1R15B-siRNAs and the control siRNA.
Supporting Information Table S2). Off-target effects were excluded with testing additional siRNAs (Figs. 2b and 2d). Furthermore, we observed increased caspase 3/7 activity by PPP1R15B silencing (Fig. 2c), indicating that downregulation of PPP1R15B induces apoptosis.

**Impairment of cell cycle progression in ERα-positive breast cancer cells by PPP1R15B deficiency**

We examined the cell cycle status of MCF-7 cells using flow cytometry after transfection with siRNAs. As shown in Figure 3a, a higher proportion of cells in G0/G1 phase was observed among PPP1R15B-silenced cells than among control cells (58 vs. 50%; p < 0.05). This was accompanied by significantly lower percentages of PPP1R15B-deficient cells than control cells in the S and G2/M phases (4.8 vs. 16.8% [p < 0.0009] and 13 vs. 28.0% [p < 0.0006], respectively). Moreover, PPP1R15B knockdown resulted in more PPP1R15B-deficient than control cells in the sub-G1 phase (24 vs. 4.9%; p < 0.003) representing increased apoptotic cells, which corroborates the caspase 3/7 activity data. We also tested the effect of PPP1R15B silencing on the number of cells in S phase through cytometric evaluation of EdU incorporation into DNA during active DNA synthesis. The rate of cell proliferation decreased to about 50% in absence of PPP1R15B (Fig. 3b). These data, together with the higher proportion of PPP1R15B-deficient than control cells in G0/G1 phase, confirmed that the G1/S cell cycle transition is impaired in PPP1R15B-deficient cells, which eventually undergo apoptosis.

We extended our analysis of cell proliferation to five additional breast cancer cell lines: T47D, BT-474, MDA-MB-231, SK-BR-3 and EVSAT.12 Despite successful PPP1R15B knockdown by siRNAs in all the cell lines (Supporting Information Fig. S1), significant reduction in cell proliferation was observed only in MCF-7, T47D and BT-474 cells compared to negative controls (p < 0.05) (Fig. 3c). Because these three cell lines are ERα-positive whereas MDA-MB-231, SK-BR-3 and EVSAT cells lack detectable ERα protein expression,13 this observation indicates a relationship between the function of PPP1R15B and presence of ERα in breast cancer cells.

Next, we assayed the abundance of the G1/S phase-specific regulatory proteins cyclin D1 and phosphorylated retinoblastoma (p-Rb) at Ser807/81114 in MCF-7 and MDA-MB-231 cells, representing ERα-positive and -negative cells, respectively. As shown in Figure 3d, we observed potent reduction of both p-Rb and cyclin D1 levels by PPP1R15B knockdown only in MCF-7 cells. With respect to the critical role of cyclin D1 in mammalian physiology and pathology,15 these data support the notion that this molecule may mediate the
observed contribution of PPP1R15B to proliferation of ERα-positive breast cancer cells.

**PPP1R15B is required for ERα abundance and transcriptional activity in breast cancer cells**

Our *in vitro* analyses indicated that the effect of PPP1R15B on breast cancer cell proliferation is correlated with the presence of ERα in the cells. We therefore sought to determine whether PPP1R15B expression differs among intrinsic subtypes of breast cancer. Indeed, analysis of gene expression profiles from the Thuerigen *et al.* dataset revealed higher levels of *PPP1R15B* mRNA expression in luminal (ERα-positive) and normal-like subtypes than in basal and Her-2-positive (ERα-negative) subtypes (Supporting Information Fig. S2). Moreover, we observed significantly higher levels of *PPP1R15B* mRNA expression in ERα-positive tumors (*p < 0.001*, two-sided *t*-test) (Fig. 4a). Consequently, we studied the effect of PPP1R15B deficiency on ERα expression and signaling in MCF-7 cells. As shown in Figure 4b, endogenous ERα protein expression decreased substantially upon PPP1R15B knockdown. We further investigated the regulatory role of PPP1R15B on ERα transcriptional activity using reporter assays. Figure 4b demonstrates that suppression of PPP1R15B by siRNAs resulted in a potent reduction of ERE-luciferase activity (threefold of control, *p < 0.05*), indicating disruption of downstream signaling via effectors of ERα, such as cyclin D1 (Fig. 3d).18,19 Taken together, these data demonstrated that loss of PPP1R15B function decreases ERα expression and transcriptional activity.

Figure 4. PPP1R15B expression correlates with ERα expression and plays a role in tamoxifen resistance. (a) The microarray dataset from Thuerigen *et al.* was used to build sample groups according to presence or absence of ER as assessed by immunohistochemistry. A direct comparison revealed *PPP1R15B* mRNA expression levels to be significantly higher in ERα-positive breast tumors than in ERα-negative samples (**p = 0.0001**). Depicted values represent log2 ratios of tumor sample against human universal reference RNA (Stratagene, La Jolla). (b) Western blot analysis of ERα protein expression in MCF-7 upon PPP1R15B silencing (upper graph) and measurement of ERα transcriptional activity using luciferase reporter assays (bottom graph) (*p < 0.05*). (c) Kaplan–Meier graphs showing a trend to (upper graph, Chanrion *et al.*19) and a significant association with better prognosis (bottom graph, Ma *et al.*20) in patients with low PPP1R15B mRNA expression after tamoxifen therapy in two independent studies (*p = 0.108* and *p = 0.031*, log-rank test). DSF: disease-free survival (in month). (d) Sensitization of 4-hydroxytamoxifen-resistant MCF-7 cells to this agent by PPP1R15B depletion as measured using cell viability assay. The effect of PPP1R15B siRNA on the ERα protein expression level was examined using Western blot analysis.
Correlation of low PPP1R15B expression with better outcome of tamoxifen therapy

As it has been reported that downregulation of ER\(\alpha\) expression sensitizes breast cancer cells to treatment with tamoxifen,\(^{20}\) we next asked whether PPP1R15B, as a regulator of ER\(\alpha\), is involved in the response of ER\(\alpha\)-positive breast cancer to this treatment. We used expression profiles available from two independent studies\(^{16,17}\) to determine whether PPP1R15B expression is associated with outcome after tamoxifen-based therapy. Low PPP1R15B expression levels correlated with improved disease-free survival durations in both studies, with a significant difference in the study by Ma and colleagues, in which, in contrast to the other study, patients received tamoxifen as monotherapy (\(p = 0.031\), log-rank test) (Fig. 4c).

Sensitization of resistant breast cancer cells to tamoxifen treatment by PPP1R15B depletion

We next tested the effect of PPP1R15B depletion by RNAi on our tamoxifen-resistant MCF-7 cell sub-line.\(^{21}\) Cells silenced for PPP1R15B showed a smaller fraction of surviving cells relative to control cells in the presence of 4OH-tamoxifen (Fig. 4d). Thus, these findings demonstrated that PPP1R15B inhibition reverses acquired resistance to tamoxifen in vitro. In addition, Western blot analysis revealed a strong reduction in ER\(\alpha\) expression following PPP1R15B knockdown in tamoxifen-resistant MCF-7 cells (Fig. 4d). Given that depletion of ER\(\alpha\) affects tamoxifen sensitivity in MCF-7 cells,\(^{20}\) our data suggest that sensitization to tamoxifen induced by PPP1R15B silencing may be mediated by reduced ER\(\alpha\) expression.

Our findings are of clinical relevance as patients with ER\(\alpha\)-positive breast cancer can benefit from targeted therapy with tamoxifen, whereas resistance to this therapy develops in many cases.\(^{72}\) Here, we show that high PPP1R15B expression was associated with shorter disease-free-survival durations in patients who underwent tamoxifen-based therapy and that suppression of PPP1R15B expression restored sensitivity to tamoxifen in a resistant cell line model of breast cancer. Most recently, Mendes-Pereira et al. reported on a genome-wide short hairpin RNA screen showing that PPP1R15B knockdown sensitized MCF-7 cells to short-term tamoxifen-based treatment.\(^{20}\) These data are very much in line with our findings. However, the involvement of PPP1R15B in long-term treatment of breast cancer cells with tamoxifen is likely closer to the scenario of resistance development in patients. Finally, our findings suggest that PPP1R15B-targeted therapy for breast cancer may help overcome tamoxifen resistance and reveal a novel regulator of ER\(\alpha\) signaling in breast cancer cells.

Acknowledgements

The authors thank Dorothee Nickles and Michael Boutros for the actin-RL luciferase construct and siRNAs and Doris Mayer for the ERE luciferase construct. The authors are also grateful to Lukas Brenner for technical help and Donald R. Norwood from the department of scientific publications, The University of Texas M.D. Anderson cancer center for editorial assistance.

References