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## Down-regulation of program cell death 4 (PDCD4) is associated with aromatase inhibitor resistance and a poor prognosis in estrogen receptor-positive breast cancer

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### Abstract

**Purpose**—Progression or recurrence due to resistance to aromatase inhibitors (AIs) is a significant clinical problem for a considerable number of patients with breast cancer. Program cell death 4 (PDCD4), a tumor suppressor protein, is targeted for degradation during tumor progression. We aimed to examine PDCD4 expression and regulation in AI-resistant breast cancer cells and association with survival in estrogen receptor (ER)-positive breast cancer patients.

**Methods**—We determined PDCD4 expression levels in AI-resistant breast cancer cell lines and ER-positive breast cancer tumors, investigated the regulation of PDCD4 in AI-resistant breast cancer cell lines, and carried out a Kaplan-Meier survival analysis in two independent cohorts that included a total of 420 patients with ER-positive breast cancer.

**Results**—PDCD4 expression was down-regulated in AI-resistant breast cancer cells, and this down-regulation was inversely correlated with activation of HER2 signaling. Moreover, lower expression of PDCD4 was significantly associated with HER2 positive status in ER-positive breast tumors. Down-regulation of PDCD4 was mediated through up-regulation of HER2 via the mitogen-activated protein kinase (MAPK), protein kinase B (PKB/AKT), and miR-21 in AI-resistant breast cancer cells. miR-21 inhibitor and fulvestrant induced PDCD4 expression and decreased cell proliferation in AI-resistant breast cancer cells. Furthermore, forced overexpression of PDCD4 resensitized AI-resistant cells to AI or hormone deprivation. Finally, we identified that down-regulation of PDCD4 was associated with a lower rate of disease-free survival in ER-positive breast cancer and higher histologic grade of breast tumors.

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#### Declaration of interest

The authors declare that there is no conflict of interest.

**Conclusions**—Expression of PDCD4 is down-regulated by HER2 signaling in AI-resistant breast cancer cells. Down-regulation of PDCD4 is associated with AI resistance and a poor prognosis in patients with ER-positive breast cancer.

## Keywords

PDCD4; aromatase inhibitor; HER2; miR-21; breast cancer; prognosis

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## 1. Introduction

Endocrine therapy plays a central role in the treatment of patients with estrogen receptor (ER)-positive advanced breast cancer. In postmenopausal patients, selective aromatase inhibitors (AIs) (i.e., letrozole, anastrozole, and exemestane), which block the peripheral conversion of androgens into estrogens and reduce the estrogen levels, are used as first-line treatment [1]. Unfortunately, resistance to AI treatment is present in a considerable number of patients, who may have no response to AIs (primary or *de novo* resistance) or will eventually relapse despite an initial response (acquired resistance) [1].

There is increasing evidence suggesting that cross-talk between the ER and the human epidermal growth factor receptor 2 (HER2) signaling is involved in the development of resistance to AIs.

ER can interact with and activate HER2 and its downstream signaling intermediates, such as the mitogen-activated protein kinase (MAPK) and protein kinase B (PKB/AKT) [2]. On the other hand, activation of HER2 signaling pathway, including the MAPK and phosphatidylinositol 3'-kinase (PI3K)/AKT, can phosphorylate and activate ER in a ligand-independent manner [3,4], which has been implicated in endocrine therapy resistance [5–7]. In patients treated with AIs, HER2 signaling is often up-regulated in breast tumors [8]. These findings suggest that targeting the ER pathway may lead to the up-regulation of HER2 pathway due to the extensive cross-talk, and ultimately results in endocrine resistance.

Program cell death 4 (PDCD4) is a tumor suppressor protein that was originally found to be induced by apoptosis [9,10]. PDCD4 binds to the translation initiation factor eIF4A and inhibits its RNA-helicase activity, thus inhibiting protein translation [11,12]. PDCD4 has been linked to tumorigenesis and tumor progression [13,14], and its expression is decreased in several type of cancers, including lung, colon, liver and breast cancers [15–18]. Like the well-known tumor suppressor phosphatase and tensin homolog (PTEN) [19], PDCD4 has been established as an important functional target of the oncogenic microRNA, miR-21, which is commonly up-regulated in solid tumors and contributes to the down-regulation of PDCD4 [20,21].

Translational aberrations are known to be associated with a poor prognosis in hormone receptor-positive breast cancer [22]. The expression of PDCD4, a translation inhibitor, is decreased during the progression of many cancers. We thus hypothesized that PDCD4 could be down-regulated during the development of AI resistance in breast cancer. In the current study, we found that PDCD4 expression was down-regulated by HER2 signaling in AI-

resistant breast cancer and associated with survival outcomes in patients with ER-positive breast cancer.

## 2. Materials and Methods

### 2.1. 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 [23–25]. Details of culture conditions are provided in the Supplementary Appendix.

### 2.2. Antibodies and reagents

Antihuman PDCD4 (#9535), PTEN (#9188), p-HER2 (Tyr1248) (#2247), p-MAPK (#9101), MAPK (#9102), p-AKT (Ser473) (#9271), AKT (#9272), GAPDH (#2118) antibodies were obtained from Cell Signaling Technology. Antihuman HER2 antibody (#06–562) was from Abcam Inc. Antihuman ER $\alpha$  (HC-20) antibody (sc-543) was from Santa Cruz Biotechnology. The p44/42 MAPK siRNA (#6560) was obtained from Cell Signaling Technology. The nontargeting control siRNA (sc-37007) was from Santa Cruz Biotechnology. The AKT inhibitor MK-2206 was obtained from Selleck Chemicals. The ER antagonist fulvestrant was from Sigma-Aldrich. The anti-miR-21 inhibitor and inhibitor negative control were purchased from Ambion.

### 2.3. Plasmid construct

The DNA fragment coding human *PDCD4* was generated by RT-PCR using RNA from MCF7 cells as the template and the following oligonucleotides as the PCR primers (forward primer: 5'-CTGGGATCCCACCATGGATGTAGAAAATGAGCAG-3'; reverse primer: 5'-GGGGCTAGCTCAGTAGCTCTCTGGTTTAAGACG-3'). The interest fragment was then cloned into the mammalian expression vector pMG-H2 using Bgl II-Nhe I restriction sites. The insert was verified by DNA sequencing.

### 2.4. Western blotting

Western blotting was performed as previously described [26]. Relative expression of proteins was normalized against the internal control GAPDH. Details are provided in the Supplementary Appendix.

### 2.5. Real-time PCR

The human *PDCD4* gene was amplified using the forward primer 5'-ACAGGTGTATGATGTGGAGGA-3' and the reverse primer 5'-TTCTCAAATGCCCTTTCATCAA-3' (PrimerBank ID 313760536c3) [27]. The *ACTB* ( $\beta$ -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. Experimental details are provided in the Supplementary Appendix.

## 2.6. Transfection

Details of transfection of cells with small interfering RNA (siRNA), estrogen responsive element (ERE) reporter plasmid, and human *PDCD4* construct are provided in the Supplementary Appendix.

## 2.7. Luciferase reporter assay

Details of luciferase reporter assay are provided in the Supplementary Appendix.

## 2.8. Cell proliferation assay

Cell proliferation was measured by an MTT assay. Experimental details are provided in the Supplementary Appendix.

## 2.9. Gene expression analysis

*PDCD4* differential expression analysis was performed in The Cancer Genome Atlas (TCGA) cohort in breast cancer patients with ER and HER status [28]. The differential expression *p*-values were determined via t-test using R [29] (<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 [30,31]. 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 [29]. Details are provided in the Supplementary Appendix.

## 3. Results

### 3.1. Down-regulation of *PDCD4* expression is inversely correlated with activation of HER2 signaling in AI-resistant breast cancer cells and ER-positive breast cancer tumors

*PDCD4*, a tumor suppressor protein, has been linked to tumor progression [14]. To test our hypothesis that *PDCD4* is down-regulated when breast cancer becomes resistant to AIs, we evaluated the mRNA and protein levels of *PDCD4* in LET-R, a MCF7aro-derived breast cancer cell line resistant to the AI letrozole, and LTEDaro, a MCF7aro-derived breast cancer cell line after long-term estrogen deprivation. We found that both the mRNA and protein levels of *PDCD4* were significantly decreased in LET-R and LTED cells, as compared with the MCF7aro parental cells (Fig. 1a–b). In contrast, the levels of PTEN, another tumor suppressor protein, were not significantly changed in LET-R or LTEDaro cells (Fig. 1b).

Because the up-regulation of HER2 signaling pathway, including the MAPK and PI3K/AKT, has been implicated in endocrine therapy resistance [5–7], we sought to determine whether *PDCD4* expression is correlated with HER2 signaling in AI-resistant breast cancer cells. To investigate the activation state of the HER2 pathways in our LET-R and LTEDaro cell lines, we evaluated the levels of phosphorylated and total HER2, MAPK and AKT by Western blotting. As shown in Fig. 1b, total HER2 expression and phospho-HER2,

phospho-MAPK and phospho-AKT were increased in both LTEDaro and LET-R cells as compared with MCF7aro cells, which were inversely correlated with the PDCD4 expression.

To further determine the correlation between PDCD4 and HER2 signaling pathways, we performed a differential expression analysis for *PDCD4* in 406 patients with ER-positive breast cancer in the TCGA cohort [28], including 318 patients with ER-positive and HER2-negative (ER+ and HER2-) breast tumors, and 88 patients with ER-positive and HER2-positive (ER+ and HER2+) breast tumors. We found that lower expression of *PDCD4* was significantly associated with HER2 positive status ( $p = 0.0002$ , fold change = 1.4), supporting that down-regulation of PDCD4 is inversely correlated with the levels of HER2 in ER-positive breast cancer (Fig. 1c).

### 3.2. Up-regulation of HER2 and MAPK signaling inhibits PDCD4 expression in AI-resistant breast cancer cells

Given the inverse correlation between low PDCD4 expression and HER2 levels in both ER-positive breast cancer patients and AI-resistant breast cancer cells, we sought to determine whether up-regulation of the HER2 pathway decreases the expression of PDCD4. We thus did comparisons between MCF7aro and HER2aro, a MCF7 derived breast cancer cell line overexpressing HER2 and a model for primary resistance to AIs [25]. We found that HER2 expression and the phosphorylation of MAPK and AKT were significantly increased, whereas both the PDCD4 protein and mRNA levels were significantly decreased in HER2aro cells as compared with MCF7aro cells (Fig. 2a–b), indicating that up-regulation of the HER2 signaling down-regulates PDCD4 expression.

To further investigate whether the MAPK pathway is involved in the regulation of PDCD4, we determined whether reduction of MAPK expression by short interfering RNA (siRNA) would alter the expression of PDCD4. We found that knockdown of MAPK significantly increased PDCD4 expression in both HER2aro and LTEDaro cells (Fig. 2c–d), suggesting that PDCD4 is down-regulated by the MAPK pathway.

### 3.3. Up-regulation of AKT inhibits PDCD4 expression in AI-resistant breast cancer cells

Protein kinase S6K1, a downstream mediator of the PI3K/AKT pathway, promotes the phosphorylation-dependent degradation of PDCD4 [32]. Because the AKT signaling pathway was up-regulated in AI-resistant breast cancer cells, we hypothesized that up-regulation of AKT signaling could reduce the expression of PDCD4. To test this hypothesis, we did comparisons between MCF7aro and AKTaro, a MCF7 derived breast cancer cell line overexpressing AKT and a model for primary resistance to AIs [25]. We found that the phosphorylation of AKT was significantly increased, whereas both the protein and mRNA levels of PDCD4 were significantly decreased in AKTaro cells as compared with MCF7aro cells (Fig. 3a–b), suggesting that activation of AKT down-regulates PDCD4 expression.

To further confirm that AKT down-regulates PDCD4, we determined whether inhibition of AKT increases the expression of PDCD4. We found that treatment with MK-2206, an AKT inhibitor, significantly increased PDCD4 expression in both AKTaro and LTEDaro cells (Fig. 3c–d), confirming that PDCD4 is down-regulated by AKT.

### 3.4. Down-regulation of PDCD4 is associated with up-regulation of miR-21 in AI-resistant breast cancer cells

PDCD4 has been established as a target of the oncogenic microRNA, miR-21, whose expression is up-regulated by HER2 [20,21,33]. High miR-21 expression is found to be associated with HER2 positive status in primary breast tumors [34]. As HER2 is up-regulated in AI-resistant breast cancer cells, we wondered whether miR-21 is increased in these cells. We thus evaluated the levels of miR-21 in MCF7aro, HER2aro, LET-R, and LTEDaro cells by quantitative real-time PCR. We found that the levels of miR-21 were significantly increased in HER2aro, LET-R and LTEDaro cells as compared with MCF7aro cells (Fig. 4a).

Next, to investigate whether PDCD4 is a target of miR-21 in AI-resistant breast cancer cells, we determined whether inhibition of miR-21 increases the expression of PDCD4 in LET-R and LTEDaro cells. MiR-21 inhibitor significantly decreased the miR-21 levels in both LET-R and LTEDaro cells (Fig. 4b). After the treatment with miR-21 inhibitor, PDCD4 levels were remarkably elevated in both LET-R and LTEDaro cells (Fig. 4c–d). Interestingly, the levels of PTEN, another target of miR-21 [19], were not changed (Fig. 4c–d), suggesting that PDCD4 rather than PTEN is a target of miR-21 in AI-resistant breast cancer cells.

### 3.5. Forced overexpression of PDCD4 resensitizes AI-resistant breast cancer cells to AI or hormone deprivation

Activation of HER2 signaling pathway can lead to the activation of ER in a ligand-independent manner [3,4]. Many groups, including us [24,35,36], have shown that ER $\alpha$  is up-regulated and constitutively activated in AI-resistant breast cancer cells, especially in LTEDaro that has a low level of PDCD4 (Fig. 5a). To test whether ER $\alpha$  plays a role in the down-regulation of PDCD4 in AI-resistant breast cancer cells, we examined the effect of fulvestrant treatment on PDCD4 expression in LTEDaro cells. Fulvestrant treatment resulted in abolished ER $\alpha$  transcriptional activity, confirming that ER $\alpha$  in LTEDaro cells responds to fulvestrant (Fig. 5b). After treatment with fulvestrant, the expression of PDCD4 was significantly increased (Fig. 5c), which is consistent with a previous report [37], indicating a role for PDCD4 in the treatment of AI-resistant breast cancer.

Because PDCD4 is a target of miR-21, we determined the effect of fulvestrant on miR-21 expression. We found that miR-21 levels were increased in LTEDaro cells following fulvestrant treatment (Fig. 5d), suggesting that the induced expression of PDCD4 by fulvestrant is not through miR-21, and that there may be a compensational increase of miR-21 after fulvestrant treatment.

As miR-21 was up-regulated in AI-resistant breast cancer cells and its expression was further increased following fulvestrant treatment, we sought to examine the effect of miR-21 inhibitor or a combination of miR-21 inhibitor and fulvestrant in AI-resistant breast cancer cells. We found that miR-21 inhibitor treatment alone decreased cell proliferation in LTEDaro cells (Fig. 5e). Combination treatment of miR-21 inhibitor and fulvestrant further decreased cell proliferation in LTEDaro cells, in an additive manner (Fig. 5e).

Next, to test whether increasing the expression of PDCD4, which is induced by miR-21 inhibitor and fulvestrant treatment, can resensitize AI-resistant breast cancer cells to AI, we generated a pMG-H2-PDCD4 expression construct. LET-R and LTEDaro cells were transfected with pMG-H2-PDCD4 expression construct or empty vector (Fig. 5f). We found that forced overexpression of PDCD4 resensitized LET-R and LTEDaro to letrozole and hormone deprivation, respectively (Fig. 5g). These data strongly suggest that PDCD4 plays a role in AI resistance of breast cancer.

### 3.6. Down-regulation of PDCD4 is associated with a poor prognosis in ER-positive breast cancer

To analyze the clinical relevance of PDCD4 down-regulation in ER-positive breast cancer, we carried out a Kaplan-Meier survival analysis. Because data on disease-free survival in the TCGA cohort are lacking, we did the analysis in other two independent cohorts that included a total of 420 patients with ER-positive breast cancer [30,31]. This analysis showed that patients with a low level of *PDCD4* expression had a worse prognosis than patients with a high level of *PDCD4* expression. A Kaplan-Meier analysis of disease-free survival among 211 ER-positive breast cancer patients in the Ivshina cohort [30] showed that higher *PDCD4* expression conferred a significant survival advantage ( $p = 0.03$ ) (Fig. 6a). Similarly, lower *PDCD4* expression was associated with a poor disease-free survival among 209 patients in the Wang cohort [31] with ER-positive breast tumors ( $p = 0.0004$ ) (Fig. 6b). These data suggest that PDCD4 expression has a prognostic value for patients with ER-positive breast cancer.

Next, we sought to determine the correlation between PDCD4 and histologic grade of tumors. Data on tumor grade are available in the Ivshina cohort [30] but not in the Wang cohort [31]. We thus performed a differential expression analysis for PDCD4 in 289 patients in the Ivshina cohort, including 68 patients with Elston grade 1 tumors, 166 with Elston grade 2, and 55 with Elston grade 3. The analysis showed a lower level of *PDCD4* in patients with high grade (Elston grade 3) tumors than in patients with low grade (Elston grade 1) tumors ( $p = 4.62e-08$ , fold change 1.5) (Supplementary Fig. S1).

In summary, our study shows that PDCD4 expression is in part down-regulated by HER2 via the MAPK, AKT and miR-21 in AI-resistant breast cancer cells. Down-regulation of program cell death 4 (PDCD4) is associated with AI resistance and a poor prognosis in ER-positive breast cancer (Fig. 6c).

## 4. Discussion

Progression or recurrence due to resistance to AIs is a significant clinical problem for a considerable number of ER-positive breast cancer patients. In this study, we found that the tumor suppressor PDCD4 was down-regulated in AI-resistant breast cancer cells. We also identified an association between the down-regulation of PDCD4 and the up-regulation of HER2 signaling in AI-resistant breast cancer cells and ER-positive breast tumors. More importantly, a poor prognosis was found in ER-positive breast cancer patients with a low level of PDCD4, further sustaining a potential contribution of PDCD4 to the development and/or outcome of AI resistance in breast cancer.

PDCD4 has been known as a tumor suppressor [38]. PDCD4 inhibits protein translation by binding to the translation initiation factor eIF4A and suppressing its activity [11,12]. PDCD4 is targeted for degradation during tumor progression [14]. Breast cancer may progress or relapse because of resistance to AIs, and we demonstrated that PDCD4, a tumor suppressor, was down-regulated in AI-resistant breast cancer cells, and that forced overexpression of PDCD4 resensitized AI-resistant cells to AI or hormone deprivation. Furthermore, our survival analysis suggests that the down-regulation of PDCD4 is clinically significant in breast cancer, since ER-positive breast cancer patients with a low level of *PDCD4* tend to have a lower rate of disease-free survival based on microarray datasets in two independent patient cohorts [30,31] that included a total of 420 patients. Consistent with our finding that low *PDCD4* predicts a poor prognosis, Meric-Bernstam et al. have reported that low protein expression of PDCD4 is associated with worse overall survival but not recurrence-free survival in hormone receptor-positive breast cancer with the use of reverse phase protein arrays in 190 patients [22]. In addition, our study showed that lower *PDCD4* was significantly correlated with high histologic grade of breast tumors. Identification of reliable biomarkers that can be used to predict patient outcomes ensure more effective clinical management, and we propose that PDCD4 is such a biomarker in ER-positive breast cancer.

Up-regulation of HER2 signaling pathway has been found in AI-resistant breast cancer cells [5,6] and breast tumors of patients treated with AIs [8]. HER2 signaling promotes cell proliferation and survival through the MAPK and PI3K/AKT pathways [39]. In our study, we found an inverse correlation between the PDCD4 expression and the activation of HER2 signaling pathways in AI-resistant breast cancer cells. Consistently, our differential gene expression analysis in the TCGA patient cohort [28] showed that lower expression of PDCD4 was significantly associated with HER2 positive status in patients with ER-positive breast cancer. We further demonstrated that HER2 down-regulated PDCD4 via the MAPK, AKT and miR-21 in AI-resistant breast cancer cells. These findings strongly suggest that the down-regulation of PDCD4 is, at least in part, mediated through HER2 in AI-resistant breast cancer. In addition to HER2 signaling, other growth factor receptors, such as the insulin-like growth factor 1 receptor (IGF1R) [40,41] and epidermal growth factor receptor (EGFR) [42], have been associated with AI resistance. As these growth factor receptors can also activate the MAPK and PI3K/AKT [43], further studies are needed to investigate whether they are involved in the down-regulation of PDCD4.

Up-regulated and constitutively activated ER $\alpha$  has been shown to play a role in AI resistance [24,35,36]. PDCD4 can be induced by anti-estrogen treatment [37]. Consistently, our results showed that PDCD4 expression was significantly increased following fulvestrant treatment in AI-resistant breast cancer cells, suggesting that ER $\alpha$  may play a role in the down-regulation of PDCD4. The mechanisms of ER $\alpha$  regulation of PDCD4 might be complicated. We evaluated the effect of fulvestrant on miR-21 expression. Interestingly, we found that fulvestrant increased the expression of miR-21 rather than decreased it, which is consistent with a report showing that estradiol represses the expression of miR-21 by activating ER in MCF7 cells [44]. These findings suggest that the regulation of PDCD4 by ER $\alpha$  is not through miR-21 and that there are other mechanisms responsible for it. One



possibility is that ER $\alpha$  activation can activate PI3K/AKT/p-70S6K signaling [45] and even can directly transcriptionally up-regulate expression of S6K1 [46], which promotes the phosphorylation-dependent degradation of PDCD4 [32]. Our results showed that activation of AKT down-regulated PDCD4 expression in support of this possible mechanism. In addition, activating mutations of *ESR1*, which encodes ER $\alpha$ , have been identified in hormone-resistant metastatic breast cancer [47,48]. It is possible that ER $\alpha$  mutant signaling may also play a role in the regulation of PDCD4, but we do not know whether it is involved in our AI-resistant models. Therefore, the mechanisms of ER $\alpha$  regulation of PDCD4 need to be further investigated.

Down-regulation of PDCD4 is often associated with up-regulation of the oncogenic microRNA miR-21 [20,21]. High miR-21 expression is associated with HER2 positive status in primary breast tumors[34], and overexpression of HER2 significantly induces miR-21 in breast cancer cells [33]. Consistent with the up-regulation of HER2 signaling, we found an increase of miR-21 levels in AI-resistant breast cancer cells. miR-21 has also been linked to MAPK or PI3K/AKT pathways [49,50], and the two major pathways can be activated by HER2. There is increasing evidence suggesting that microRNAs are involved in AI resistance [51–53], including miR-21, which has been shown to have elevated expression following combine treatment of tamoxifen and exemestane [52]. In this study, we found that treatment with miR-21 inhibitor remarkably increased the expression of PDCD4 and decreased cell proliferation in AI-resistant breast cancer cells. Furthermore, we showed that forced overexpression of PDCD4 resensitized AI-resistant cells to AI or hormone deprivation. These data suggest potential roles of miR-21 and PDCD4 in AI resistance of breast cancer.

In conclusion, our study shows that the tumor suppressor PDCD4 expression is down-regulated by HER2 signaling in AI-resistant breast cancer cells. The down-regulation of PDCD4 is associated with AI resistance and may predict a poor prognosis in patients with ER-positive breast cancer.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>AI</b>	aromatase inhibitor
<b>AKT/PKB</b>	protein kinase B
<b>ER</b>	estrogen receptor
<b>FBS</b>	fetal bovine serum

<b>HER2</b>	human epidermal growth factor receptor 2
<b>HR</b>	hormone-receptor
<b>MAPK</b>	mitogen-activated protein kinase
<b>MEM</b>	minimal Eagle's medium
<b>PDCD4</b>	Program cell death 4
<b>PI3K</b>	phosphatidylinositol 3'-kinase
<b>PTEN</b>	phosphatase and tensin homolog
<b>siRNA</b>	short interfering RNA

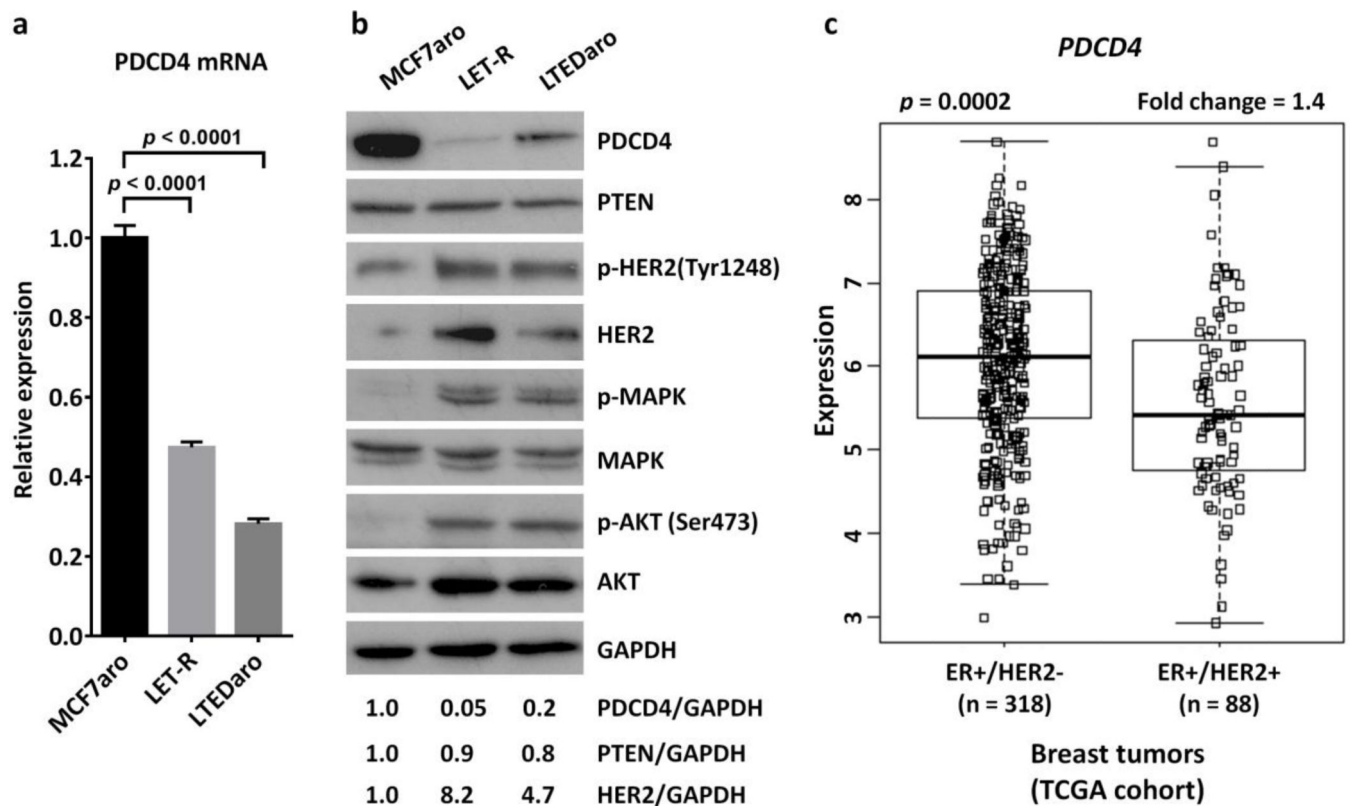
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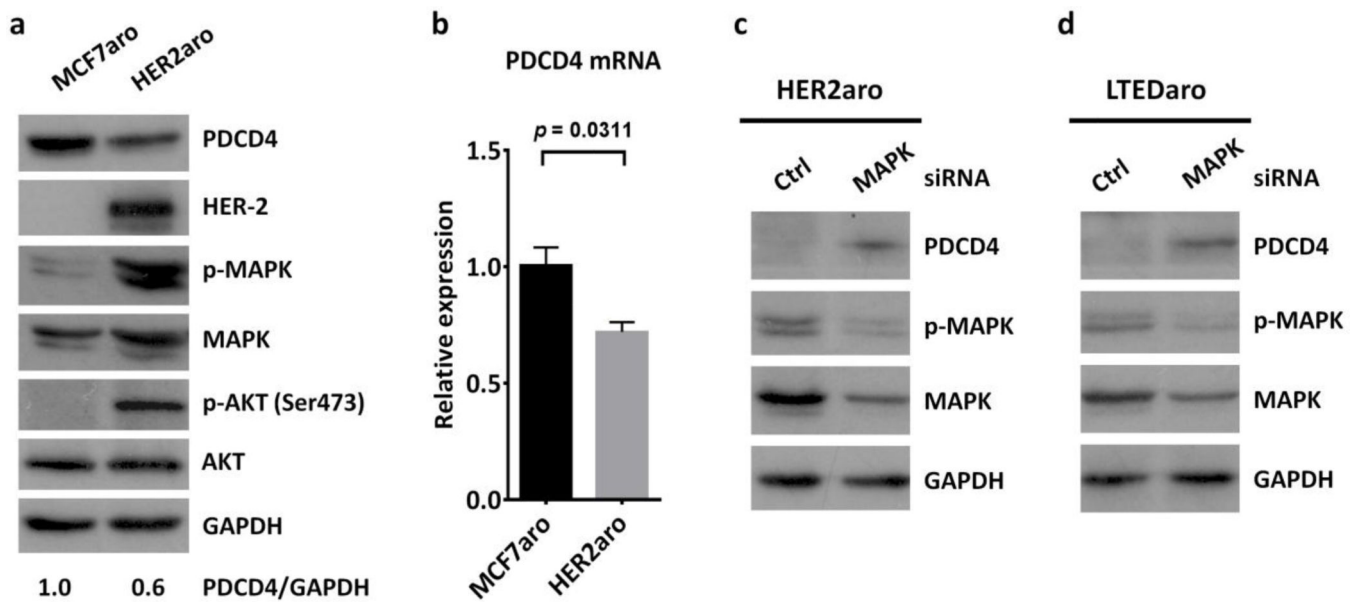


**Fig. 1. Down-regulation of PDCD4 is inversely correlated with activation of HER2 signaling in aromatase inhibitor-resistant breast cancer cells and ER-positive breast cancer tumors**

a. Real time PCR analysis of *PDCD4* expression in MCF7aro, LET-R and LTEDaro cells cultured in defined culture media described in the Materials and Methods. Values were normalized to *ACTB* and plotted relative to the expression of the MCF7aro cells. Data are means  $\pm$  SE (n=3).

b. Western blotting analysis of PDCD4, PTEN and the activation of the HER2, MAPK and AKT signaling pathways in MCF7aro, LET-R and LTEDaro cells. The quantification of PDCD4 was normalized to the loading control GAPDH, then normalized to the MCF7aro cells.

c. Gene expression analysis in 406 patients with ER-positive breast cancer in the TCGA cohort. Differential expression analysis for *PDCD4* shows a lower level of *PDCD4* in patients with ER+ and HER2+ breast tumors than in patients with ER+ and HER2- breast tumors.



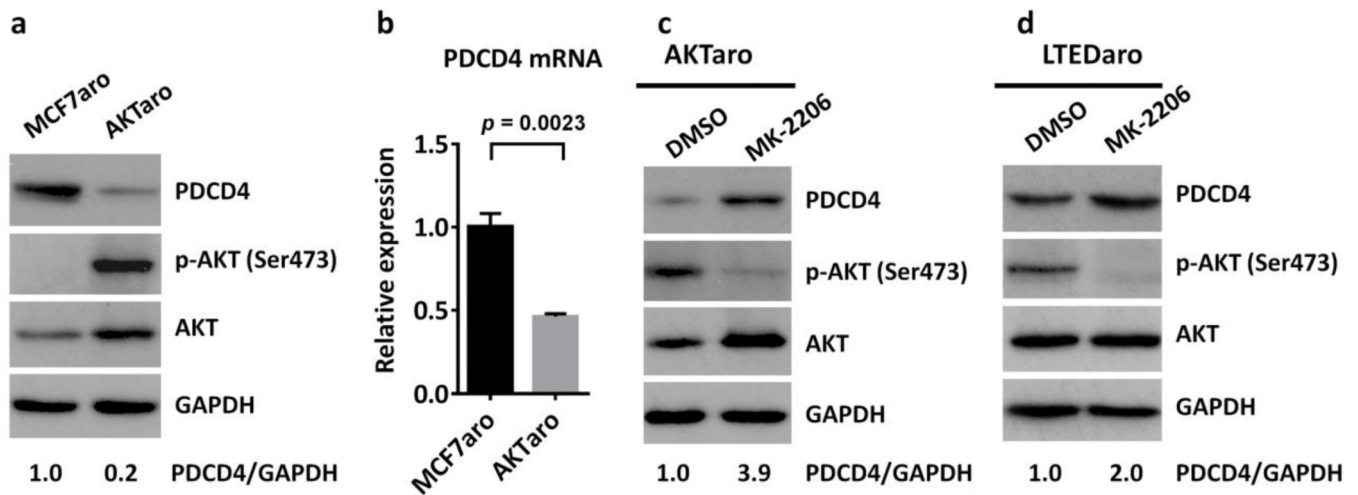
**Fig. 2. Up-regulation of HER2 and MAPK signaling inhibits PDCD4 expression in aromatase inhibitor-resistant breast cancer cells**

a. Western blotting analysis of PDCD4 expression and the activation of the HER2 signaling pathway in MCF7aro and HER2aro cells cultured in defined culture media. The quantification of PDCD4 and HER2 was normalized to GAPDH, and then normalized to the MCF7aro cells.

b. Real time PCR analysis of *PDCD4* expression in MCF7aro and HER2aro cells. Values were normalized to *ACTB* and plotted relative to the expression of the MCF7aro cells. Data are means  $\pm$  SE (n=3).

c. PDCD4 protein levels in whole-cell lysates from HER2aro cells transfected with a nontargeting control (Ctrl) siRNA or a siRNA targeting MAPK. The quantification of PDCD4 was normalized to GAPDH, then normalized to the MCF7aro cells.

d. PDCD4 protein levels in whole-cell lysates from LTEDaro cells transfected with a nontargeting control (Ctrl) siRNA or a siRNA targeting MAPK. The quantification of PDCD4 was normalized to GAPDH, then normalized to the control siRNA



**Fig. 3. Up-regulation of AKT inhibits PDCD4 expression in aromatase inhibitor-resistant breast cancer cells**

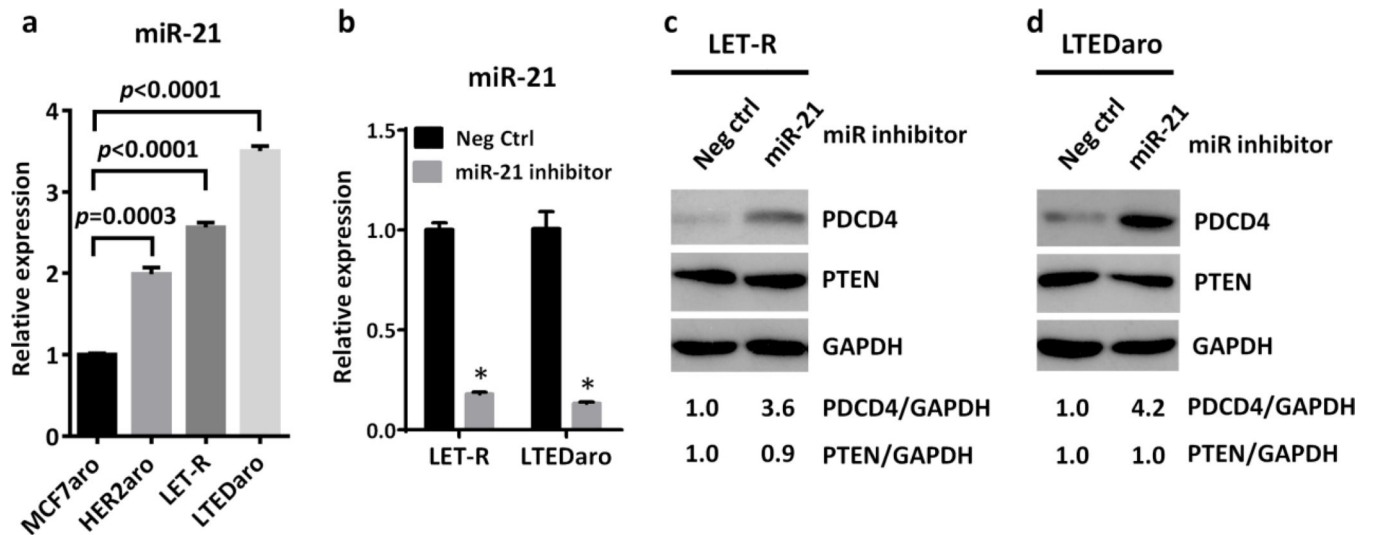
a. Western blotting analysis of PDCD4 expression and the activation state of AKT signaling in MCF7aro and AKTaro cells cultured in defined culture media. The quantification of PDCD4 was normalized to GAPDH, then normalized to the MCF7aro cells.

b. Real time PCR analysis of *PDCD4* expression in MCF7aro and AKTaro cells. Values were normalized to *ACTB* and plotted relative to the expression of the MCF7aro cells. Data are means  $\pm$  SE (n=3).

c. PDCD4 protein levels in whole-cell lysates from AKTaro cells treated with DMSO or MK-2206 1 $\mu$ M for 24 hr. The quantification of PDCD4 was normalized to GAPDH, then normalized to the control siRNA.

d. PDCD4 protein levels in whole-cell lysates from LTEDaro cells treated with DMSO or MK-2206 1 $\mu$ M for 24 hr. The quantification of PDCD4 was normalized to GAPDH, and then normalized to the control siRNA





**Fig. 4. Up-regulation of miR-21 inhibits PDCD4 in aromatase inhibitor-resistant breast cancer cells**

a. Real time PCR analysis of miR-21 expression in MCF7aro, HER2aro, LET-R and LTEDaro cells cultured in defined culture media. Values were normalized to U6 RNA and plotted relative to the expression of the MCF7aro cells. Data are means  $\pm$  SE (n=3).

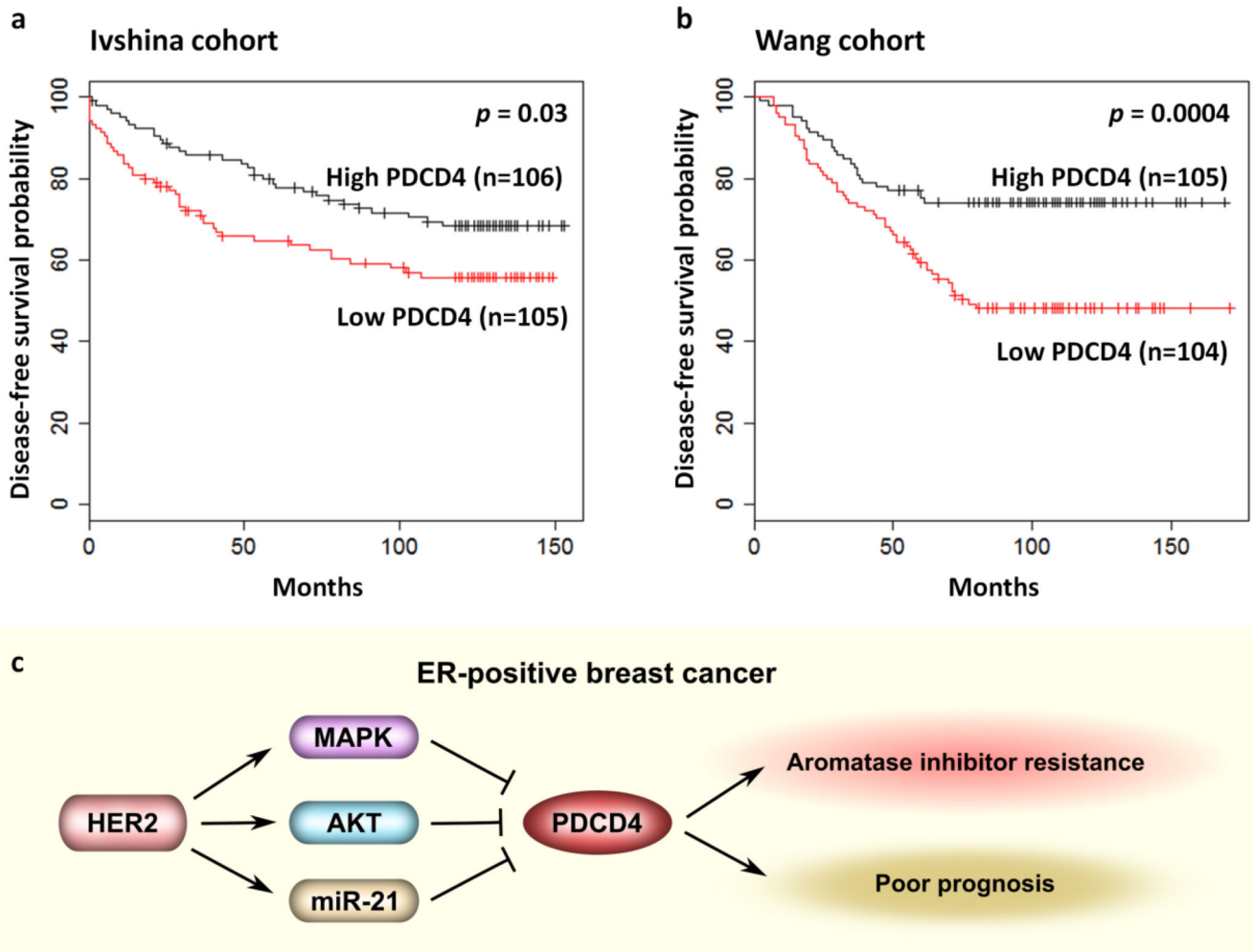
b. Real time PCR analysis of miR-21 expression in LET-R and LTEDaro cells transfected with an inhibitor negative control (Neg Ctrl) or a miR-21 inhibitor for 48 hr. Values were normalized to U6 RNA and plotted relative to the expression of the inhibitor negative control. Data are means  $\pm$  SE (n=3). Asterisk (\*) denotes  $P < 0.0005$  (t test).

c. Western blotting analysis of PDCD4 and PTEN expression in LET-R and LTEDaro cells transfected with an inhibitor negative control (Neg ctrl) or a miR-21 inhibitor for 72 hr. The quantification of PDCD4 was normalized to GAPDH, then normalized to the control siRNA



f. PDCD4 protein levels in whole-cell lysates from LET-R and LTEDaro cells transfected with pMG-H2-PDCD4 expression construct or empty vector for 72 hr.

g. Cell proliferation of LET-R and LTEDaro cells transfected with pMG-H2-PDCD4 expression vector or empty vector. MTT was measured after 72 hr. Data are means  $\pm$  SE (n=3).



**Fig. 6. Poor prognosis predicted by low expression of *PDCD4* in ER-positive breast cancer**

a. Kaplan-Meier estimates of disease-free survival are shown for 211 patients with ER-positive breast cancer in the Ivshina cohort. The curves show a lower rate of disease-free survival with low-*PDCD4* breast tumors.

b. Kaplan-Meier estimates of disease-free survival are shown for 209 patients with ER-positive breast cancer in the Wang cohort. The curves show a lower probability of disease-free survival with low-*PDCD4* breast tumors.

c. Proposed model of *PDCD4* down-regulation by HER2 in ER-positive breast cancer.