Tyrosine Phosphorylation of p27Kip1 Correlates with Palbociclib Responsiveness in Breast Cancer Tumor Cells Grown in Explant Culture

Susan R.S. Gottesman1, Jonathan Somma2, Vladislav Tsiperson3, Lisa Dresner4, Usha Govindarajulu5, Priyank Patel6, and Stacy W. Blain3

Abstract

Cdk4-targeting drugs, such as palbociclib, are approved for metastatic ER/PR+, Her2- breast cancer. However, other than loss of retinoblastoma, which is very rare in this subset, there are no biomarkers to predict response. Cyclin D or cdk4 levels are not by themselves indicative, because p27Kip1 is required for cyclin D–cdk4 complex activation. Tyrosine phosphorylation of p27, including modification on residue Y88 (pY88), activates DK4–p27, and the pY88 level correlates with palbociclib responsiveness in cell lines. We developed dual IHC staining for p27 and pY88, and found that benign breast epithelium was negative, while breast cancer biopsies (of varied hormonal status) could be stratified for pY88 status. Lack of pY88 suggested that DK4 was inactive, and that these samples would not have the target required for palbociclib response. Tumor resection material was grown in explant culture, treated with palbociclib, and stained with Ki67 as a marker of response. Explants from the no pY88 group were nonresponsive, while explants from the low or high pY88 group responded to drug.

Implications: Use of the pY88 biomarker, as a surrogate for cdk4 activity, may identify patients responsive to cdk4-targeting drugs and expand use of this therapy.

Visual Overview: http://mcr.aacrjournals.org/content/molcanres/17/3/669/F1.large.jpg.

Introduction

Cyclin D–cdk4/6 complexes are critical for the G1–S-phase transition and subsequent cell-cycle progression (1). This complex is stabilized by the assembly factor p27Kip1, and then activated by tyrosine phosphorylation of p27 on residue Y88 (or Y89; ref. 2). pY88 p27–cyclin D–cdk4 is the active or open complex, competent to phosphorylate the G1 gatekeeper protein, retinoblastoma (Rb), and cause passage into S-phase. p27 is critical, but inhibitory, for the activity of another cell-cycle cyclin-dependent kinase, cdk2, a molecule normally required for full passage into S-phase. Although both Y phosphorylated and nonphosphorylated p27 interact with cdk2 and inhibit this kinase, the pY88 form is preferentially degraded, leading to p27-free cdk2 and cdk2 activation (3). Thus, p27 plays a pivotal role...
at several steps in the cell cycle: Y phosphorylated p27 directly activates cdk4 and indirectly increases cdk2 activation.

Cyclin D–cdk4/6 is activated in many human cancers, either by overexpression of cyclin D, or loss of the CDKN2A tumor suppressor gene, which encodes p16ink4A, the cdk4/6 inhibitor (1). These observations prompted development of specific cdk4 inhibitors (cdk4i), such as palbociclib, abemaciclib, and ribociclib, which have been approved in combination with estrogen modulation therapy for patients with metastatic, relapsed, or drug refractory ER/PR+, Her2− breast cancer (1, 4, 5). However, data from clinical trials suggest that approximately 20% of patients exhibit primary resistance to these drugs, and that even initial responders will develop secondary resistance within 2 years of treatment, resulting in the clinical observation of increased progression-free but no overall survival benefit (6). Unlike most other targeted therapies, there are no clinically approved companion diagnostic markers to identify patients who have the active cdk4 target and thus might respond to this therapy. While loss of RB expression correlates with palbociclib nonresponsiveness in tissue culture cells, this is a relatively rare event in estrogen receptor–positive (ER+) breast cancer and has not been validated clinically. Several other cell-cycle proteins, such as cyclin E, cdk2, and p16 have also been implicated, but again none have shown clinical utility. Previous work suggested that compensatory activity of cdk2 may explain acquired secondary resistance to palbociclib, while the activity of the cyclin D–cdk4 complex itself may predict primary responsiveness (7).

Whereas overall cdk4 or cyclin D levels have been shown not to be predictive, we showed that the level of pY88-p27 correlates with active cdk4 and palbociclib responsiveness in tissue culture cells (3). We hypothesized that the level of phosphorylated p27 would correlate with active cdk4 and therefore could be used to predict responsiveness to palbociclib in patients. By double staining for p27 and pY88 (with an antibody specific for Y88) using immunohistochemistry (IHC), we show that both breast cancer cell lines and human breast cancer biopsy material, can be divided into groups based on the level of pY88 staining and that this grouping corresponded with palbociclib responsiveness in ex vivo short term cultures of fresh tumor. We suggest that pY88 staining can be used as a marker to identify tumors with the active cdk4 target and to predict response to cdk4i.

**Materials and Methods**

**Archival breast tissue**

Archival tissue blocks were obtained, with IRB approval, from the files of SUNY Downstate Medical Center, University Hospital of Brooklyn (Brooklyn, NY). Breast specimens, both benign and malignant, were obtained by core needle biopsy. Hormone status of tumors was determined as part of clinical workup.

**Explant culture**

Patients were consented prior to surgery in accordance with SUNY Downstate Medical Center IRB requirements. Fresh breast tumor tissue samples (6 mm × 1 mm × 1 mm) were obtained within 20 minutes of removal of tumor from patients and placed into 3 ml of complete culture media (RPMI1640 with 10% FBS, 1% penicillin–streptomycin, 10 μg/ml insulin, and 1 mg/100 ml hydrocortisone). Samples were dissected into approximately 1-mm3 pieces, and placed (one specimen per sponge) onto hemostatic gelatin dental sponges (Vetspen, Elanco), which had been previously hydrated for 2 hours in 12-well cell culture dishes in 1.5 ml complete media at 37°C and 5% CO2. Explants were maintained in complete media for 60 hours prior to treatment. Media was removed and 1.5 ml fresh media containing 100 nmol/L or 500 nmol/L palbociclib, or an equal volume of DMSO, was added to each well. Culture plates were incubated for 48 hours. Each tumor specimen was treated in duplicate per condition. Samples were then removed from sponges, placed into embedding cassettes, and fixed for 24 hours in 10% neutral buffered formalin followed by paraffin embedding.

**IHC**

**Antibodies.** Ki-67 (SC-23900, Santa Cruz Biotechnology); p27-Kip1 (BD Biosciences, catalog no 610242); Rab catalog no 93095, Rabser780 catalog no 9307, and cdk2T160, catalog no 2561S (Cell Signaling Technology). The p27/pY88 antibody and its specificity have been described previously (8). Paraffin-embedded tumor sections or cell blocks were cut into 5-μm sections. MCF7-ALT cells, which express ALT in the presence of doxycycline (ClonTech) were grown and treated ±doxycycline as described previously (3). Cell blocks were used as a control in cdk2T160 IHC. Ki67 staining was performed in either the automated stainer (Ventana Medical Systems, Inc.) for the archival material or manually as described below for the explant material. Slides were incubated for 30 minutes at 65°C, deparaffinized, and rehydrated. Endogenous peroxide activity was quenched by incubation with peroxidase solution for 30 minutes at room temperature. Slides were washed three times in 1× TBST, followed by antigen retrieval in 1× target retrieval solution (DAKO, S-1659) for 30 minutes in 100°C water bath. Following antigen retrieval, slides were washed in 1× PBS, incubated with protein block for 1 hour (DAKO, 090930-2), incubated overnight at 4°C with the respective antibodies, and developed using the Multiview IHC Kit (ENZO, ADI-950-101-0001).

**p27/pY88 dual staining assay.** Slides were incubated overnight with pY88 antibody. The next day, sections were washed in 1× TBST and then subjected to antigen retrieval as described above, followed by incubation with p27-Kip1 antibody overnight at 4°C and developed as described.

**Microscopic evaluation**

Analysis of hematoxylin and eosin–stained sections including grading according to Modified Bloom Richardson (MBR) score was carried out blindly by two independent pathologists. For IHC stains, on patient material, four to six such high-power fields (400 ×) were evaluated where possible and then averaged for a total percent positive/tumor sample. For explant samples with fewer viable tumor cells, total viable nuclei over the entire slide were counted and percent positive was reported. Samples were generally run three separate times for each stain and replicate reading were performed blindly at different times. Any discrepancies were reviewed jointly and resolved. Positive and negative controls were analyzed with each staining.

**Statistics**

In Figs. 2 and 3, means and SE were calculated using Excel and plotted. In Fig. 3C (right), raw Ki67 values from the 1, 2 pY88+ group and the 0 pY88+ group were analyzed using a box and whisker plot using GraphPad Prism. A two-sided paired t test was performed in Fig. 3 to compare the Ki67 scores from the pY88
group 1, 2 and pY88 group 0 patient populations. We used the 0.05 level of significance.

Results

We had previously shown that the level of pY88 p27 measured by immunoblot analysis correlated with the level of active cdk4 measured by in vitro kinase assay and with palbociclib responsiveness (3). The MCF7 breast cancer cell line, which responds to low concentrations of palbociclib (IC\textsubscript{50} = 200 nmol/L), had low, but detectable levels of pY88 and active cdk4, while HCC1954 cells had more pY88, more active cdk4 and required a higher concentration of palbociclib to reach IC\textsubscript{50} (1,000 nmol/L; ref. 3).

We developed a dual pY88/p27 IHC assay, where pY88 stained pink and p27 stained brown, for use with formalin-fixed, paraffin-embedded archival material from patients at University Hospital (Figs. 1 and 2). Benign material obtained from stereotactic core breast biopsies in patients without risk factors for breast cancer served as nonneoplastic control (Figs. 1A and 2A, light tan box) and was compared with core needle biopsies from patients with invasive ductal carcinoma, not otherwise specified (Figs. 1B–D, 2A). For the three breast cancer subgroups [ER/PR\textsuperscript{+}/Her2\textsuperscript{−}, Her2\textsuperscript{+}, and triple negative (TN)], Ki67 positivity ranged from 10% to 50% and grading ranged from I to III using MBR score with 50% being grade II (Fig. 2A). We scored for the percent of viable tumor cells that were pY88\textsuperscript{−}. While strong p27 staining (brown) was detected in normal epithelial cells, all benign epithelium was negative for pY88 (pink staining) (Figs. 1A and 2A), consistent

Figure 1.

Dual pY88/p27 IHC assay with FFPE archival human breast tissue. A, Benign breast. B, ER\textsuperscript{+}/PR\textsuperscript{+}/Her2\textsuperscript{−} breast cancer; C, Her2\textsuperscript{+} breast cancer; and D, ER\textsuperscript{−}/PR\textsuperscript{−}/Her2\textsuperscript{−} (TN) breast cancer. All were invasive ductal breast carcinoma. Material was stained to determine % viable tumor cells positive. Score: 0, 0%–5%; 1, 5%–25%; and 2 ≥25%.
with observations that cdk4 is inactive and benign mammary cells
are not in cycle. The breast cancer samples, with the exception of
D40, all had high p27 expression (>20% cells positive), consistent
with rare loss of this protein in breast cancer (Fig. 2A). Using the
criteria of % pY88 cells, three groups were identified in the breast
cancer samples. In Group 0, <5% pY88 cells were detected, with
staining resembling benign material (Fig. 1B, representative
D21; Fig. 2A, light green box). This suggests that this group does
not have detectable levels of active cdk4 and might not respond
to palbociclib treatment. The rest of the samples had cells that
stained with pY88. In Group 1, between 5% and 25% of cells
stained for pY88 (Fig. 1B, representative D18; Fig. 2A, dark
green box). In Group 2, >25% of cells stained pY88 (Fig. 1C,
representative D23; Fig. 2A, yellow box). When the data was
grouped according to pY88 staining subgroups in Fig. 2A, we
found that stratification was independent of overall MBR or Ki67
status (Fig. 2A). Roughly similar percentages of pY88 group 0, 1,
and 2 were seen in ER/PR/HER2, HER2, and TN samples: 20–
25% scored as 0 (no pY88 cells), 25%–30% scored as 1, and 50%
scored as 2 (high % of pY88 cells; Fig. 2B). This suggests that
20–25% of patients across subgroups did not have detectable
levels of the active cdk4 target and would never respond to
palbociclib treatment. To validate the reproducibility of the
pY88/p27 staining protocol, we stained material from 6 patients
three independent times, and the mean ± SE is plotted (Fig. 2C).
We found that whereas the intensity of the stain did vary (data not
shown), the percentage of pY88 cells was consistent across the
replicates, with relatively small SE. In addition, in all repeated
cases, the pY88 groupings (Groups 0, 1, or 2) did not change
during replicate testing (Fig. 2C).

At the time of this study, few patients at University Hospital had
been treated with palbociclib, and outcome data was not yet
available. We therefore identified patients scheduled to undergo
mastectomy or lumpectomy (Fig. 2D). Following informed
content, we analyzed their corresponding archival biopsy material.
Three patients from the cohort were excluded because there was
only in situ carcinoma (R6, R9, and R11). ER/PR/HER2, HER2,
and TN subgroups are all represented in this cohort (Fig. 2D, top).
In 8 of 10 cases, initial biopsies were analyzed. The other 2
patients (R10 and R13) were recurrences. For patient R10, a core
biopsy, which documented the recurrence, was used for IHC and
markers of palbociclib response, including cyclin E, cdk2, or RB
processing or the presence of too few tumor cells in the individual
explant piece, which precluded accurate analysis. In all cases, the
explant material was compared with the biopsy material for
morphologic similarities.

The Ki67 status of the biopsy material was compared with the
Ki67 staining seen in the DMSO-treated explants to determine
whether similar proliferation rates were seen in tumor removed
from the patient and immediately fixed and that grown for several
days in explant culture (Fig. 3B). Ki67 levels varied across the
patient cohort but in general, the level in the biopsy was com-
parable with the level seen in the DMSO-treated explants, suggest-
ing that the explants resembled the parent tumor. For example,
the explant showing the lowest level of Ki67 (R7) was from the
same patient showing the lowest Ki67 staining in biopsy material
(Fig. 3C, right). Representative Ki67 staining for explant samples
from R10 (Group 2) and R13 (Group 0) are shown (Fig. 3B).
Ki67 staining decreased for R10 as the palbociclib concentration
increased, while staining did not vary for R13. All of the Ki67
values for each treatment condition (DMSO, 100 and 500 nmol/L
palbociclib) for all of the 1, 2 pY88 scoring explants (R1, R12, R8,
R10, R7, and R3) or the 0 pY88 scoring explants (R4, R2, R13, and
R5) were combined and analyzed using a box and whisker plot
(Fig. 3C, right). Each box represents up to 36 data points for pY88
score 1, 2 patients or up to 24 for pY88 score 0 patients. Decreases
seen in Ki67 staining after palbociclib treatment in the 1, 2 pY88
scoring group were statistically significantly, while the smaller
changes seen with the four explants from Group 0 were not
different statistically.

Thus, pY88 status correlated with palbociclib response in the
explant culture assay as determined by Ki67 levels. It has been
suggested that several other cell-cycle proteins might serve as
markers of palbociclib response, including cyclin E, cdk2, or RB
(10). We stained the biopsy material from our cohort with antibodies
to antibodies against total RB, RbSer780 (phosphorylated RB), or
cdk2T160 (the catalytically active form of cdk2), and a represen-
tative staining is shown (Fig. 3D). With the exception of 1 patient
(R13), all of the samples were positive for RB and RB phosphor-
ylation, although the percentage of RB and phosphorylation
varied among patients (Fig. 3D, right top). R13 had a pY88 score
of 0, and the corresponding explant material was not inhibited
by palbociclib, consistent with the RB status of this biopsy.
Significantly, R5, R2, and R4, which were RB and RbSer780-
positive, also had pY88 scores of 0 and were nonresponsive to
palbociclib in the explant assay. As these patients were ER/PR/
HER2, they would normally be clinical candidates for palbociclib
if they progressed to the metastatic setting due to their RB
status. Our results suggest that they would not be responsive to
palbociclib, and that pY88 staining can be used to predict this
nonresponsiveness.

All of the samples were also stained with cdk2T160 (Fig. 3D).
The cdk2T160 antibody was shown to be specific for active
cdk2 by staining cell blocks generated from MCF7–alt cells grown
± doxycycline (Fig. 3D, right, bottom). In the presence of
500 nmol/L and at a lower, more pharmacologically relevant
concentration of 100 nmol/L. After 48 hours of treatment, the
explants were removed from culture, formalin-fixed, and paraffin-
embedded (FFPE). In total, six explants were grown per patient
duplicates for each of the three conditions) and each explant was
stained for Ki67 as a marker of proliferation at least two times,
to provide N ≥ 4 per condition, which were read blindly on different
days (Fig. 3B). The exceptions to this were due to tissue loss during
processing or the presence of too few tumor cells in the individual
explant piece, which precluded accurate analysis. In all cases, the
explant material was compared with the biopsy material for
morphologic similarities.

Gottesman et al.
Figure 2.
A, Analysis of archival material from 37 patients with breast cancer from all subgroups (ER+/PR+/Her2+, top; Her2+, middle; and ER+/PR-/Her2+, bottom), stained with the pY88/p27 dual assay, and then grouped into different pY88 scores (light green = 0, dark green = 1, and yellow = 2). Samples were also scored for Ki67 and MBR (tubular + nuclear + mitotic rate, respectively, defined by the WHO). B, Summary of subgroup cohorts. C, Six samples from A were stained in triplicate as independent runs and read independently to determine reproducibility of the pY88/p27 stain and marked with */C3 in A. Top, p27 staining and bottom, pY88 staining. Data, mean */C6 SE. D, Patient cohort with pY88/p27. Neoadjuvant therapy: 1 = Adriamycin–cytoxan 4 cycles; 2 = Taxol 4 cycles; and 3 = Pertuzumab, trastuzumab, 4 cycles. Biopsies from this cohort were analyzed in the pY88/p27 IHC assay (n ≥ 3 independent stains). Data are mean ± SE. Left, p27 staining (brown). Right, pY88 staining (pink). Line depicts break between pY88 subgroup 1 and 2.
Figure 3.
A. Procedure for explant experiment. N indicates the number of times each sample was independently stained. B, Patient cohort analysis with Ki67 staining. Biopsy material (blue); fresh material grown in explant culture treated with DMSO (red). Data are mean +/− SE, N ≥ 3. C, Left, representative patient material (R10, R13) grown in explant culture treated with DMSO, 100 nmol/L, or 500 nmol/L palbociclib for 48 hours, FFPE, and stained with Ki67. Right, box and whisker plot of the Ki67 staining for the combined different treatment groups for the pY88 0 (R2, R4, R13, and R5) and R1, R12, R7, R8, R10, and R3) subgroups. P values for each comparison based on paired t test. D, Left, representative biopsy material from R1, R2, and R13 stained with Rb, RbSer780, and Cdk2T160 staining. NA, tissue no longer available. Yellow box, pY88 group 1, 2; green box, pY88 group 0. Rb and RbSer780 staining: +, <10%; ++, >10%. Cdk2T160 staining: +, >5% staining. N = 2. Right, bottom, MCF7-ALT cells treated ± doxycycline (Dox) to induce ALT and inhibit cdk2, used as a control for cdk2T160 staining. +Doxycycline condition, cells are arrested (data not shown).
doxycycline and ALT induction, we had previously shown that cdk2 was inactive in *in vitro* kinase assay and cdk2T160 was not detected by immunoblot analysis (3). The IHC results were consistent. Although staining levels varied, no correlation between the amount of cdk2 activity seen in biopsy material and palbociclib response could be seen. Both explants that responded to palbociclib and those that did not had active cdk2.

**Discussion**

pY88 status correlated with palbociclib response in the short-term explant culture assay as determined by Ki67 level changes, suggesting that analysis of pY88 status could predict the presence of the active cdk4 target, which is required for palbociclib response. Examination of active Cdk2, Rb or Rb phosphorylation by IHC did not correlate with palbociclib response, suggesting that pY88 status was more predictive or that IHC was not sensitive enough to separate differences. TN and Her2 patients, two populations in which palbociclib is not currently used, were present in this cohort and responded similarly, suggesting that some patients in these subgroups do have the active cdk4 target and might show response to drug. Palbociclib should inhibit any cdk4-dependent tumor of any primary site, especially those with strong oncogenic signaling, such as Her2 overexpression, and use of a biomarker could identify patients who might be responsive. Gong and colleagues identified that cancer cell lines that expressed cyclin D–activating features were more sensitive to abemaciclib inhibition (11). These features include cyclin D1, D2, and D3 alterations that increase cyclin D stability and/or expression and ultimately cyclin D–cdk4/6 activity, and we would predict many or most of these cell lines might also be positive in the pY88 assay. Interestingly, they identified these alterations in cell lines from multiple types of cancer where cdk4i has not been extensively studied, consistent with the idea that having a biomarker for cdk4i response would permit use of cdk4i outside of the breast cancer space. Unlike most other targeted therapies used clinically, cdk4i, like palbociclib, are prescribed without any indication that the target of the therapy (active cdk4) is present in the patient. While our study suggests that pY88 may be a marker for the active cdk4 target, the same way Her2 staining identifies tumors that may respond to trastuzumab, we did not address whether it would be predictive for response in patients with metastatic breast cancer receiving combination therapy of palbociclib and letrozole. The presence of the target and long-term clinical response to the drug are two separate, but linked, issues, and this study only addresses the former. We use the pY88 biomarker to identify active cdk4 in the explants and then demonstrate that this information is sufficient to predict response to the drug. Comparison of pY88 status in biopsy material with outcome data from patients treated clinically with cdk4i will be required to determine whether pY88 can also serve as a clinical biomarker of cdk4i response.

**Disclosure of Potential Conflicts of Interest**

Pai is a research scientist at Concarlo Holdings LLC. S.W. Blain has ownership interest (including stock, patents, etc.) in Concarlo Holdings, LLC. No potential conflicts of interest were disclosed by the other authors.

**Authors' Contributions**

Conception and design: S.R.S. Gottesman, J. Somma, V. Tsiperson, L. Dresner, S.W. Blain

Development of methodology: S.R.S. Gottesman, J. Somma, V. Tsiperson, P. Patel, S.W. Blain

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.R.S. Gottesman, J. Somma, V. Tsiperson, T. Persing, U. Govindarajulu, S.W. Blain

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.R.S. Gottesman, J. Somma, V. Tsiperson, U. Govindarajulu, S.W. Blain

Writing, review, and/or revision of the manuscript: S.R.S. Gottesman, J. Somma, V. Tsiperson, U. Govindarajulu, S.W. Blain

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Somma, V. Tsiperson, P. Patel

Study supervision: J. Somma, V. Tsiperson, S.W. Blain

**Acknowledgments**

The authors thank Khurram Shafique and Mengru Li for expert technical assistance. This work was supported by NIH R01CA201536 to S.W. Blain. This work was independently verified by the SUNY Compliance Management Committee. This work was supported by NIH R01CA201536.

Received February 23, 2018; revised October 19, 2018; accepted December 12, 2018, published first December 17, 2018.
Molecular Cancer Research

Tyrosine Phosphorylation of p27Kip1 Correlates with Palbociclib Responsiveness in Breast Cancer Tumor Cells Grown in Explant Culture

Susan R.S. Gottesman, Jonathan Somma, Vladislav Tsiperson, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-18-0188

Visual Overview
A diagrammatic summary of the major findings and biological implications:
http://mcr.aacrjournals.org/content/17/3/669/F1.large.jpg

Cited articles
This article cites 11 articles, 4 of which you can access for free at:
http://mcr.aacrjournals.org/content/17/3/669.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://mcr.aacrjournals.org/content/17/3/669.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mcr.aacrjournals.org/content/17/3/669.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.