Targeting Tyrosine Phosphorylation of PCNA Inhibits Prostate Cancer Growth

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Abstract

The proliferation cell nuclear antigen (PCNA) is a critical protein required for DNA replication in proliferating cells including cancer cells. However, direct inhibition of PCNA in cancer cells has been difficult due to the lack of targetable sites. We previously reported that phosphorylation of tyrosine 211 (Y211) on PCNA is important for the proliferative function of PCNA when this protein is associated with the chromatin in cancer cells. Here, we show that the Y211 phosphorylation of PCNA is a frequent event in advanced prostate cancer. To explore the potential of this signaling event in inhibition of cancer cell growth, we used a synthetic peptide, the Y211F peptide, which when present inhibits phosphorylation of Y211 on endogenous PCNA. Treatment with this peptide, but not a scrambled control peptide, resulted in S-phase arrest, inhibition of DNA synthesis, and enhanced cell death in a panel of human prostate cancer cell lines. In addition, treatment with the Y211F peptide led to decreased tumor growth in PC3-derived xenograft tumors in nude mice. Our study shows for the first time that PCNA phosphorylation at Y211 is a frequent and biologically important signaling event in prostate cancer. This study also shows a proof of concept that Y211 phosphorylation of PCNA may be used as a therapeutic target in prostate cancer cells, including cells of advanced cancers that are refractory to standard hormonal therapies. Mol Cancer Ther; 10(1); 29–36. ©2011 AACR.

Introduction

Prostate cancer is the most frequent cancer occurring in men in the United States and is the second leading cause of cancer deaths in men. It is estimated that in 2009, 192,280 new cases of prostate were diagnosed, and that 27,360 of these patients would succumb to this disease (American Cancer Society, Cancer Facts and Figures, 2009). Progression of prostate cancer follows a relentless pattern. During early-stage growth, cancer cells depend on androgen and, therefore, are sensitive to antiandrogen therapy. However, as the disease progresses, the tumor becomes resistant to androgen depletion and resumes active cell proliferation in the face of androgen deprivation. Currently, there is no cure for androgen-independent prostate cancer. In addition, a substantial proportion of patients with primary lesions localized to the prostate gland when first diagnosed can develop incurable disseminated disease after local therapy (1). Thus, there is a substantial need for new therapies that may target prostate cancer and the progression of this disease.

Proliferating cell nuclear antigen (PCNA) is the molecular coordinator for DNA replication and for maintaining genome integrity (2–10). PCNA forms a homotrimeric sliding clamp that encircles the chromatin and acts as a molecular platform to recruit proteins involved in DNA synthesis, cell-cycle control, and DNA damage response, and repair (2, 7, 10–12). Owing to its function in cell proliferation, PCNA has been widely used as a tumor marker for cancer cell progression and patient prognosis (13–25). Given its important role in the proliferation of cancer cells, which constitute the major proliferating components in cancer-bearing patients, the inhibition of PCNA may also result in suppression of cancer progression. However, the regulation of PCNA function in cancer cells is not fully understood, making it difficult to identify the appropriate molecular niche to inhibit this abundant nuclear protein.

PCNA exists in 2 distinct forms: the replication-competent chromatin-bound form and the chromatin-unbound form which is not engaged in DNA synthesis (26). We previously reported that chromatin-bound...
PCNA, but not the unbound form, is phosphorylated at Y211 (phospho-Y211) by the EGF receptor (27). This phosphorylation event is upregulated during S phase of the cell cycle. Further study showed that this phosphorylation enhances PCNA activity in DNA replication and DNA damage repair partly by increasing the stability of PCNA to chromatin. Thus, Y211 phosphorylation is a potential target for the specific modulation of the proliferation-active form of PCNA.

In this study, we show that phosphorylation of Y211 is a frequent event observed in human prostate cancer. Moreover, we found that Y211 phosphorylation was inhibited by in vitro and in vivo treatment with a permeable peptide specific to the Y211 motif. Downregulation of Y211 phosphorylation in prostate cancer cells resulted in inhibition of cell growth and tumor development in a xenograft model. These results provide a proof of concept for the idea that targeting Y211 phosphorylation of PCNA can be an efficient approach to prostate cancer treatment.

Materials and Methods

Cell culture, peptides, and antibodies

The cell lines PC3, DU145, and LNCaP were purchased from American Type Culture Collection and have been characterized recently (<6 months) using short tandem repeat profiling (Johns Hopkins University Fragment Analysis Facility, Baltimore, MD). All cells were maintained in DMEM/F12 (1:1) with 10% FBS. The Y211F (Ac-CGRKKRRQRRRGTALRFLTFK-CONH₂) and Scramble (Ac-CGRKKRRQRRRGPFLTFKCR-CONH₂) peptides were synthesized at the Keck Peptide-synthesis Facility of Yale University, New Haven, CT. The following antibodies used in this study were purchased: α-tubulin (Sigma); cyclin B (BD Pharmingen), cyclin E and PCNA (Santa Cruz), phospho-Y211 PCNA (Bethyl).

Immunohistochemical staining

Prostate cancer tissue microarray (Imgenex; Catalog number IMH-303) was dewaxed by baking at 62°C for 1 hour. Antigen retrieval was done by heating with a microwave in 10 mmol/L of citrate (pH 6.0). The slides were then incubated with an anti-phospho-Y211 PCNA antibody overnight at room temperature, followed by incubation with biotin-conjugated secondary antibody. The immunocomplexes were then stained with avidin–biotin–peroxidase complex and amino-ethyl carbazole chromogen. Samples with no primary antibody or an IgG control antibody served as negative controls. The mean intensity of phospho-Y211 staining in each tissue section was obtained by multiplying the relative intensity score (0–3) by the percentage of epithelial cells staining positive for phospho-Y211.

Cell viability and proliferation analysis

A CellTiter-Glo luminescent cell viability assay kit (Promega) was used following the manufacturer's instruction to assess the effects of the treatments on cell viability. For each experiment, 1,000 to 3,000 cells per well were plated in 96-well plates in triplicate. The experiments were repeated at least 3 times. Cell proliferation was assessed using a colorimetric BrdU proliferation kit by following the manufacturer’s instructions (Roche; Catalog no. 1164729001). Briefly, cells treated with the peptides were labeled with BrdU for 3 to 4 hours. The genomic DNA was then fixed and denatured, then incubated with the peroxidase-conjugated anti-BrdU antibody for 90 minutes. The substrate of the conjugated peroxidase was then added and the reaction product was measured by the absorbance (A₄₇₀nm–A₄₉₂nm). The results were then normalized by the number of total viable cells, which was determined by a side-by-side cell viability assay as described above.

Cell-cycle and annexin V staining analyses by flow cytometry

Cells were harvested by trypsin, washed with PBS, and then fixed in 70% ethanol. The fixed cells were stained with 25 μg/mL of propidium iodide (Sigma) in the presence of 1 μg/mL RNase (Sigma). For fluorescence-activated cell sorting (FACS) analysis, data were collected using a FACS Calibur flow cytometer and analyzed by the software ModFit (Verity). The cell-cycle distribution was evaluated by counting greater than 10,000 cells per sample. For annexin V staining, cells treated with the peptides were stained with APC (adenomatous polyposis coli)-conjugated annexin V for 15 minutes in the dark before processing to flow cytometry following the manufacturer’s instructions (BD Biosciences).

Immunoprecipitation and Western blotting analysis

Cells were lysed by incubation with the NETN buffer [150 mmol/L of NaCl, 1 mmol/L of EDTA, pH 8.0, 20 mmol/L of Tris, pH 8.0, 0.5% NP-40, 25 mmol/L of NaF, 2 mmol/L of Na₃VO₄, 20 μL/mL aprotinin (Sigma), and 0.1 mol/L of PMSF]. For Western analysis, the lysates were separated in acrylamide gels, transferred to a PVDF (polyvinylidene difluoride) membrane (Bio-Rad), and probed with the indicated antibodies. Bands were visualized by a chemiluminescence-based detection method (Fisher/Pierce) that used horseradish peroxidase-conjugated secondary antibodies. For immunoprecipitation, 1 to 2 mg of protein was used for each reaction. Proteins were incubated with the antibody at 4°C overnight. Protein G agarose was then added to precipitate the antibody protein complex. The beads were then washed 4 times with NETN buffer. The immunocomplexes were then released by boiling in 2× loading buffer followed by Western blotting analysis, as described. Band intensities were quantitated by using NIH Image software.

In vivo tumor growth

PC3 cells (2.5 × 10⁶ in 100 μL of sterile Dulbecco's phosphate-buffered saline) were inoculated into nu/nu mice by subcutaneous injection into the flanks. Each
group included 4 mice inoculated with tumor cells on both flanks \((n = 8)\). When the tumors were palpable, mice were grouped randomly into 3 groups with 4 mice in each group. The mice were then treated with control vehicle, the control scramble peptide, or the Y211F peptide, by intratumoral injection. Tumor growth was monitored weekly by measuring tumor perpendicular diameters. Tumor volume \((V)\) was calculated using the following formula: 

\[ V = \text{length} \times \text{diameter}^2 \times 0.5. \]

**Statistical analysis**

Data from each assay were expressed as means \(\pm\) SD \((n = 3)\). Statistical differences between 2 groups were determined by the Student’s \(t\) test. \(P < 0.05\) was considered significantly different.

**Results**

To assess the extent of PCNA Y211 phosphorylation in prostate cancer, a tissue microarray (TMA), consisting of tumor tissue derived from human prostate cancer patients, was screened by immunohistochemical (IHC) staining using the anti-phospho-Y211 antibody. In this array, 18 of 38 (47.4%) tumors scored high for Y211 phosphorylation whereas 20 of 38 (52.6%) scored with negative to low for Y211 phosphorylation (Fig. 1 and Table 1). Although the small sample size prohibited the evaluation of whether there was a statistically significant correlation between phospho-Y211 PCNA staining and pathologic parameters, we did note that all the stage IV patients \((n = 3)\) scored high for Y211 phosphorylation.

![Figure 1](http://example.com/figure1.png)

**Figure 1.** Phosphorylation of PCNA at Y211 is frequently observed in prostate cancer. Human prostate cancer tissues were stained with an anti-phospho-Y211 PCNA antibody. A, representative micrographs of stained specimens are shown. Top, a tissue section negative for phospho-Y211 PCNA; bottom, a tissue section positive for phospho-Y211 PCNA. Bar = 0.1 mm. B, two tumor-adjacent normal tissue pairs. Pair 1, the tumor (top) and adjacent normal tissue (bottom) are both positive for phospho-Y211 PCNA staining; Pair 2, the tumor tissue is positive for phospho-Y211 PCNA staining (top) but the adjacent normal tissue is negative (bottom). Bar = 0.1 mm.

![Table 1](http://example.com/table1.png)

**Table 1.** Phospho Y211 PCNA expression in human prostate specimens

<table>
<thead>
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<th>PY211 expression</th>
<th>% samples</th>
<th>Mean intensity</th>
<th>Intensity range</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>47.4 (18/38)</td>
<td>202.5</td>
<td>120-285</td>
</tr>
<tr>
<td>Undetectable to low</td>
<td>52.6 (20/38)</td>
<td>40.75</td>
<td>0-80</td>
</tr>
</tbody>
</table>

NOTE: The percentage of human prostate specimens that stained positive for phospho-Y211 expression and the mean intensity of phospho-Y211 staining are shown. The number of phospho-Y211–expressing samples over the total number of samples examined is shown in parentheses.

Importantly, this study also showed that Y211 phosphorylation of PCNA is a frequent event in prostate cancer. Interestingly, positive phospho-Y211 staining was observed in the 6 of 7 matched adjacent normal tissues included in the TMA (examples of positive and negative staining are shown in Fig. 1). In total, our IHC study suggests that phosphorylation of PCNA at Y211 is found in about half of human prostate cancers.

We previously showed that Y211 phosphorylation can be specifically inhibited by using a synthetic peptide with the same 12 amino acid sequence derived from the proximal region of the Y211 residue of PCNA, except with the tyrosine (Y) residue replaced by a phenylalanine (F; ref. 27). The sequence was fused with the 10 amino acid TAT peptide for efficient cell transduction and nuclear entry (28, 29). To examine the function of PCNA Y211 phosphorylation in prostate cancer cells, we treated a panel of prostate cancer cells with the chimeric TAT-Y211F peptide (referred to hereafter as the Y211F peptide). As a negative control, we used the TAT peptide fused with the same amino acid residues as in the 12 amino acid Y211F sequence, but in scrambled order (referred to hereafter as the scramble peptide). Treatment with the Y211F peptide, but not with the same dose of the control scramble peptide, resulted in inhibition of Y211 phosphorylation of PCNA in the prostate cancer cells PC3, LNCaP, and DU145 (Fig. 2). Y211 phosphorylation is
known as a hallmark of proliferating cells in the S phase of the cell cycle (27). Consistent with the important function of Y211 phosphorylation of PCNA in cell growth, treatment with the Y211F peptide, but not the control scramble peptide or mock treatment, significantly inhibited growth of these prostate cancer cells in a dose-dependent manner (Fig. 3). To determine whether the Y211F peptide blocks cell growth through inhibition of PCNA Y211 phosphorylation, we tested whether the phosphomimetic mutant PCNA/Y211E could rescue the growth inhibition induced by the Y211F peptide. We reasoned that, if the Y211F peptide inhibited cell growth by inhibiting Y211 phosphorylation, cells expressing the Y211E mutant PCNA should have increased resistance to inhibition by the Y211F peptide. Indeed, PC3 cells expressing the PCNA/Y211E mutant were partially resistant to the growth inhibition effects of the Y211F peptide in comparison with cells expressing the wild-type PCNA (Fig. 3D). This result supports the idea that the effect of the Y211F peptide is specific to the phosphorylation event at Y211 of PCNA.

To further analyze the mechanism of cell growth inhibition by the Y211F peptide, prostate cancer cells treated with the peptides were subjected to flow cytometry analysis (Fig. 4). Treatment with the Y211F peptide, but not with the control scramble peptide or vehicle alone (Mock), had a prominent impact on cell-cycle progression, with an accumulation of cells in the S phase of the cell cycle in PC3 cells (Fig. 4A and Supplementary Table S1). However, the Y211F peptide-treated cells were not actively engaged in proliferation, as determined by BrdU incorporation analysis, suggesting that these cells were arrested in the S phase (Fig. 4B). Consistent with this observation, expression of cyclin E, the primary cyclin governing S-phase progression, was decreased in the Y211F peptide-treated cells, whereas expression of cyclin B, which controls the progression of the M phase of the cell cycle, was not altered by the treatment (Fig. 4C). In addition to the cytostatic effect, treatment with the Y211F peptide resulted in a significant increase in the sub-G0 fraction, an indication of enhanced apoptosis (Fig. 4D). This is consistent with an increase in the annexin V-positive cell population in response to treatment with the Y211F peptide, but not to mock treatment or treatment with the scramble control peptide (Fig. 4E). Similar effects were observed in LNCaP (Supplementary Figs. S1 and S2) and DU145 cells (Supplementary Figs. S3 and S4). In each case, as observed in PC3 cells, treatment...
with the Y211F peptide, but not the scramble peptide or mock treatment, resulted in an accumulation of cells in the S phase, accompanied by decreased cell proliferation as indicated by reduced BrdU incorporation.

Given the important role of Y211 phosphorylation in the growth function of PCNA, we asked whether the inhibition of cell growth induced by the Y211F peptide in cultured cancer cells could be recapitulated in vivo. To do this, we tested the growth-suppressing activity of the peptide in tumor xenografts derived from PC3 cells implanted subcutaneously into nude mice (Fig. 5). Treatment by the Y211F peptide, but not by the mock treatment with the vehicle alone or by the control scramble peptide, significantly suppressed tumor growth, as measured by tumor volume (Fig. 5A), as well as by the actual weight of isolated tumors (Fig. 5B and C). IHC staining using the anti-phospho-Y211 antibody confirmed the downregulation of Y211 phosphorylation of PCNA in the tumors injected with the Y211F peptide, in contrast to the tumor tissues injected with vehicle alone or the scramble peptide (Fig. 5D). These results corroborate with the in vitro data and show the importance of Y211 phosphorylation in prostate cancer cell growth as well as its potential as a target for cancer therapy.

Discussion

Enhanced proliferation provides the essential growth advantage to cancer cells of the primary tumor and at metastatic lesions. This high proliferative potential has...
been exploited as the major therapeutic target by conventional chemotherapy, which is the most commonly used frontline therapeutic approach for many cancer types. However, chemotherapy is not often used in the treatment of prostate cancer. Part of the reason is that these genotoxic agents induce DNA damage in both normal cells and cancer cells and, therefore, can result in high nonspecific cytotoxicity, which can be particularly harmful to older patients. Thus, identifying and specifically targeting proliferative cancer cells is an important means to targeting metastatic hormone-refractory prostate cancer. We have shown previously that Y211 phosphorylation of PCNA is an essential signaling event that maintains the function of PCNA on the chromatin during DNA replication (27). The current study shows that targeting the DNA synthesis machinery by blocking Y211 phosphorylation of PCNA is a promising approach.

We show that Y211 phosphorylation of PCNA is a frequent event in advanced prostate cancer. In each of the 3 prostate cancer cell lines tested, inhibition of the phosphorylation by using a cell-permeable PCNA inhibitor peptide resulted in growth arrest accompanied by blockage of cell-cycle progression through the S phase, which was confirmed by BrdU incorporation assays. In addition, expression of the S phase indicator, cyclin E, was downregulated by the peptide. We further showed that the inhibition effect can be partially rescued by the phosphomimetic Y211E mutant of PCNA, suggesting that the growth inhibition effect of Y211F peptide is mediated through a PCNA-directed mechanism. This growth inhibition effect can be recapitulated in a xenograft tumor model in which tumor growth was significantly suppressed by the treatment of the Y211F peptide. Interestingly, in addition to the antiproliferation effect, downregulation of PCNA Y211 phosphorylation also resulted in cell death, which was shown by the sub-G0 fraction of flow cytometry and the positive annexin V staining in the treated cells. It is possible that the cytotoxicity is triggered in the arrested S-phase cells by stalked replication forks, which harbor single- and double-strand breaks in the DNA. Thus, the induction of cell death may be linked to the pathway mediating the response to DNA damage. Alternatively, Y211 phosphorylation of PCNA may have a function in regulating cell viability during cell proliferation. Discriminating between these possibilities will require further understanding of the signaling pathway leading to cell death and the underlying molecular mechanism.

Expression of Y211-phosphorylated PCNA has been associated with poor overall survival in breast cancer (27). The current study suggests that Y211 phosphorylation of PCNA may be an important tumor marker in other types of malignancy. More importantly, this is
the first study showing the potential of Y211 phosphorylation of PCNA as a promising cancer therapy target. The major challenge in prostate cancer treatment is that although the disease initially responds to andhormonal therapies, development of advanced stage with hormone-refractory tumor is a frequent and fatal outcome. Our finding that Y211 phosphorylation occurs in both hormone-dependent (LNCaP) and hormone-independent (PC3 and DU145) prostate cancer cell lines and that inhibiting Y211 phosphorylation of PCNA resulted in significant growth inhibition both in vitro and in vivo suggests that targeting Y211 phosphorylation can also be applied to the hormone-refractory prostate cancer cells. The current work is a proof of concept that targeting the Y211-phosphorylated PCNA can be an effective strategy against prostate cancer. Although the phospho-Y211-directed peptide is a rational strategy to target proliferation-competent PCNA, limitations associated with peptides as a therapeutic agent, particularly in a systemic approach, exist. Other molecular strategies, such as the conjugation of tumor-specific ligands and incorporation of structural or chemical modifications, can further improve the specificity, delivery, and stability for systemic administration. To this regard, it is noteworthy that there are numerous reported successes that provide precedent for this strategy such as the peptide inhibitor of the Jun kinase signaling pathway (30). Our results warrant further investigation to identify small molecules that can specifically target Y211 phosphorylation of PCNA. These strategies may prove to be particularly useful in targeting the proliferative potential of cells in advanced cancers that are resistant to conventional therapeutics. One concern is whether targeting PCNA, which is a ubiquitously expressed proliferation factor, could lead to severe general cytotoxicity. Our strategy targets a subpopulation of PCNA harboring a specific posttranslational modification (Y211 phosphorylation), which has been shown to be expressed in breast cancer tissues and to be significantly correlated with poor survival. In addition, cancer cells likely constitute the compartment with the highest level of cell proliferation in a cancer patient. It is therefore conceivable that targeting Y211 phosphorylation of PCNA would have a greater impact in tumor cells versus normal tissues. It should also be noted that posttranslational modification is not a requirement for PCNA to conduct DNA synthesis, as recombinant PCNA, which is believed to be devoid of such modifications, has been used for short DNA synthesis in vitro (31). Thus, it appears that posttranslational modifications of PCNA mainly serve as modulators of PCNA function (2). Indeed, our previous study showed that the function of phospho-Y211 is, at least in part, to enhance PCNA stability on the chromatin, therefore promoting its activity in DNA synthesis and DNA damage repair (27). Y211 phosphorylation may modulate different functions of PCNA during cell proliferation through other mechanisms. Together, targeting these functions is expected to have a major impact on cancer cells, which require a high level of proliferative activity to support rigorous growth. Furthermore, as many other oncogenic pathways also function in enhancing cancer growth and tumor progression, combination therapies that target these pathways along with phospho-Y211 of PCNA could be a new strategy for cancer therapy. Finally, the finding that phospho-Y211 PCNA is expressed in phenotypically normal tissues adjacent to tumors suggests that the signaling event can occur early in tumorigenesis. If this is the case, phospho-Y211 PCNA may signal increased growth advantage in otherwise normal tissues and could be used as a marker of early lesions. Further understanding the mechanisms regulating Y211 phosphorylation of PCNA in the tissues surrounding tumors may provide molecular insight into tumor development in prostate cancer that could be exploited to therapeutic advantage.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed by any authors.

Acknowledgment

The authors thank Maryellen Daston and Glenn Doerman for assistance in editing the manuscript and preparing the graphics.

Grant Support

This work was supported in part by the following grants: Susan G. Komen Research Award KG080540 (S-C. Wang), the Department of Defense Prostate Cancer Research Award PC073091 (S-C. Wang), the Marlene Harris-Bide Cincinnati Pilot Grant (S-C. Wang), PHS grant P30 DK078392 (S-C. Wang and S.E. Waltz), R01 CA109311 (M-C. Hung), D0899-011-005 (M-C. Hung), NSC-2632-B-001-MY3 (M-C. Hung), the Sister Institution Fund of China Medical University and Hospital and MDACC (M-C. Hung), and R01 CA125379 (S.E. Waltz).

Received August 24, 2010; revised October 29, 2010; accepted November 17, 2010; published online January 10, 2011.

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www.aacnjournals.org Mol Cancer Ther; 10(1) January 2011 35

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