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Molecular and Cellular Pathobiology

Cowden Syndrome-Related Mutations in PTEN Associate with Enhanced Proteasome Activity

Xin He¹, Nicholas Arrotta¹, Deepa Radhakrishnan¹, Yu Wang¹, Todd Romigh¹, and Charis Eng¹,²,³,⁴,⁵

Abstract

Germline mutations in PTEN have been described in a spectrum of syndromes that are collectively known as PTEN hamartoma tumor syndrome (PHTS). In addition to being mutated in the germline in PHTS, somatic loss-of-function PTEN mutations are seen in a wide range of sporadic human tumors. Here, we show evidence of upregulated proteasome activity in PHTS-derived lymphoblasts. PTen knock-in mice and cell lines expressing missense and nonsense PTEN mutations. Notably, elevated nuclear proteasome activity occurred in cells expressing the nuclear mislocalized PTEN-K62R mutant, whereas elevated cytosolic proteasome activity was observed in cells expressing the cytosolic-predominant mutant PTEN (M3M4 and C136R). Treatment with proteasome inhibitor MG-132 was able to restore both nonsense and missense mutant PTEN protein levels in vitro. PHTS patients with destabilizing PTEN mutations and proteasome hyperactivity are more susceptible to develop neurologic symptoms such as mental retardation and autism than mutation-positive patients with normal proteasome activity. A detailed molecular and functional analysis shows that PTEN mutants most likely cause proteasome hyperactivity via 2 different mechanisms, namely, induction of proteotoxic stress and loss of protein phosphatase activity. These results provide novel insights into the cellular functions of PTEN and reveal molecular mechanisms whereby PTEN mutations increase proteasome activity and lead to neurologic phenotypes. Cancer Res; 73(10): 3029–30. ©2013 AACR.

Introduction

PTEN is regarded as one of the most integral tumor suppressors due to its regulation of cellular proliferation, differentiation, and survival between multiple signaling pathways (1). Located at 10q23, PTEN encodes a protein that functions as a dual lipid and protein phosphatase (2, 3). Germline mutations in PTEN occur in subsets of several clinically distinct inherited disorders, such as Cowden syndrome, Bannayan–Riley–Ruvalcaba syndrome, and autism spectrum disorders (4), collectively termed PTEN hamartoma tumor syndrome (PHTS). The most common PHTS is Cowden syndrome, which is a multiple hamartoma syndrome associated with a high risk of benign and malignant tumors of the thyroid, breast, endometrium, and megencephaly (5, 6). Recently, our group reported that approximately 25% of individuals who meet the strict diagnostic criteria for Cowden syndrome, who were accrued from the community, have a germline pathogenic PTEN mutation (7).

In a wide variety of sporadic tumors, especially glioblastoma multiforme (8) and endometrial carcinoma (9), high frequencies of somatic PTEN mutations are well documented.

Certain mutations in DNA can result in misfolded or truncated proteins. Ubiquitin-dependent protein degradation is an essential mechanism of cellular clearance of such misfolded proteins. Following multiple cycles of misfolding in the endoplasmic reticulum, proteins are retrotranslocated to the cytosol and conjugated with ubiquitin. Polyubiquitinated proteins are targeted for degradation by an ATP-dependent process in proteasomes, which are located in the cytosol and nucleus (10). Using a cohort of 3042 Cowden syndrome patients, we have shown that decreased peripheral blood PTEN protein levels correlate with individuals harboring germline PTEN mutations (7). More interestingly, decreasing PTEN protein levels roughly correlate with increasing, so-called, PTEN-CC score, which is a measure of increasing phenotypic load (7). These observations may suggest that proteasome hyperactivity may play a role in the resulting Cowden syndrome phenotypes if a subset of PTEN mutations diminish PTEN’s protein stability by whatever mechanism.

Proteotoxic stress is a cellular stress that is induced by proteins that fail to fold properly. Several lines of evidence suggest that proteotoxic stress and proteasome hyperactivity may be a hallmark of human cancers (11). Indirect
evidence for this type of “gain-of-function” of proteasomes in cancers is showed by the increased sensitivity of cancer cells to proteasome inhibitors such as bortezomib (12). We therefore hypothesized that proteasome hyperactivity is a common phenomenon in cells expressing misfolded PTEN proteins encoded by mutant PTEN gene, germane to PHTS. We sought to address our hypothesis by interrogating proteasome activity in a mouse model, PHTS-derived lymphoblastoid cells and cancer cell lines expressing PTEN mutations.

Materials and Methods

Reagents

MG-132 (>99% pure) was purchased from LC Laboratories (Cat# B-1408). Cycloheximide was purchased from Sigma Aldrich. The mitogen-activated protein kinase (MAPK)/ERK kinase (MEK) inhibitor, PD98059, was purchased from Calbiochem. The AKT inhibitor, Perifosine (>99% pure), was purchased from LC laboratories.

Cell culture

MCF-7 and HEK-293 cells were grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS. Immortalized lymphoblast cells obtained from patients with PHTS or normal controls were grown in RPMI-1640 supplemented with 20% FBS.

Patients

We used peripheral blood samples and lymphoblastoid lines derived from PHTS patients with a missense mutation (C136R) and 2 common nonsense mutations (R233X and R335X) and from normal PTEN wild-type (WT) controls. Informed consent was obtained for all research participants (PHTS individuals and controls) in accordance with procedures and protocols approved by the respective Human Subjects Protection Committee of each participating institution. All research participants, whether PHTS patients or controls, participated on a voluntary (unpaid) basis.

Mutation analysis

Genomic DNA extracted from peripheral leukocytes, obtained from both PHTS patients and controls, were amplified by PCR and subjected to direct Sanger sequencing (ABI 3730xl) of all PTEN exons and flanking introns. All controls had no detectable PTEN sequence alterations.

Mutagenesis

Each PTEN mutant was engineered using the QuikChange in vitro site-directed mutagenesis system according to the manufacturer’s instructions (Stratagene). The accurate construction of mutant PTEN plasmids was verified by sequence analysis. All plasmids generated contain a FLAG-epitope at the C-terminus such that the expressed PTEN contains a C-terminal FLAG fusion.

Plasmid transfection

Cells were either grown on coverslips in 6-well plates (for confocal microscopy assays) or cultured in 60 cm² dishes (for Western analysis) and were transfected with plasmid DNA (PTEN-WT or PTEN-mutant) using FuGENE 6 (Roche Applied Science).

Protein isolation, SDS-PAGE, and Western blot analyses

For whole-cell protein lysates, cells were washed twice with ice-cold PBS and harvested in M-PER buffer (Thermo Scientific) with protease inhibitors and phosphatase inhibitors. The Mammalian Protein Extraction Reagent (M-PER) is designed to provide highly efficient total soluble protein extracts from cultured mammalian cells. After quantification, proteins were run on 4%–15% SDS–PAGE gels and transferred to nitrocellulose. Blots were probed with primary antibodies and followed by incubation with secondary antibody and then visualized using enhanced chemiluminescence detection. FLAG M2 monoclonal antibody was purchased from Sigma Aldrich. PTEN antibody (Clone 6H2.1) was from Cascade Biosciences.

Cycloheximide chase study

MCF-7 cells stably expressing FLAG-tagged WT or mutant PTEN were incubated with cycloheximide (50 μg/mL) and were harvested at the indicated time points. Whole protein lysates were extracted and ran for Western blots using anti-FLAG antibody for transfectant PTEN and GAPDH antibody as a loading control.

Quantitative reverse transcription-PCR

PTEN mRNA expression was measured by quantitative reverse-transcription PCR assay (qRT-PCR) to quantitate as described previously (13) using SYBR Green (Applied Biosystem). Expression of GAPDH was used as the internal control. PTEN Primers are as following: F: AAGGCACAAGAGGCCC-355 and 460 nm, respectively.

Proteasome activity assay

Cells or brain tissues were homogenized in lysis buffer (0.05 mol/L HEPES, 0.005 mol/L EDTA, 0.15 mol/L NaCl, 1% Triton X-100) and 3 μg whole-cell lysates (for fractionation study, 3 μg cytosolic-fraction, 10 μg nuclear-fraction), were mixed with the fluorogenic proteasome substrates Suc-LLVY-AMC (Enzo Life Sciences) and incubated in 37°C for 2.5 hours. Fluorescent values were read with excitation and emission wavelengths of 355 and 460 nm, respectively.

Subcellular fractionation

For the nuclear and cytosolic fractionation, cells were harvested by trypsinization and then were incubated with buffer A (10 mmol/L MOPS, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, and 1% Triton X-100) for 15 minutes on ice with vortex for every 5 minutes. Cells were then centrifuged at 13,000 rpm for 5 minutes at 4°C and the supernatant was carefully collected as the cytosolic fraction. The pellets were then incubated with buffer A for another 15 minutes and separated by centrifugation at 13,000 rpm for 5 minutes at 4°C. The pellets were dissolved in RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.5% Na-Deoxycholate, 1% Triton X 100). Both the cytosolic and the nuclear lysates were...
adjusted to the same concentration before loading for the proteasome activity assay. Alpha-tubulin and PARP-1 were used as loading controls for cytosolic and nuclear protein fractions, separately.

**Indirect immunofluorescence and confocal microscopy**

Cells were seeded in 6-well plates with coverslips. Cells were fixed in freshly prepared 100% methanol for 1 minute at −20°C. After 5-minute incubation with 0.3% Triton X-100 in PBS, the coverslips were washed 3 times in PBS, blocked in PBS with 10% goat serum for 30 minutes, incubated with primary antibodies for 1 hour, washed 3 times in PBS, and incubated with Alexa Fluor dye-labeled secondary antibodies for 1 hour at the concentration of 1:1,500 (Invitrogen). FLAG M2 monoclonal antibody was purchased from Sigma Aldrich. Cells were mounted on glass slides with Pro-Long Gold antifade reagent with DAPI (Invitrogen) and visualized on a Leica TCS-SP spectral laser scanning confocal microscope.

**Murine model study**

Pten*M3M4* missense knock-in–mutant mice were generated in our lab and were backcrossed more than ten times onto a CD1 genetic background. Briefly, the M3M4 double mutations were targeted into the Pten exon 7, which contains the NLS-like sequence (5). Male mouse littermates were sacrificed at 10 weeks ages; megencephalic brains were dissected out for proteasome activity assay and Western blot. All protocols involving mice were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic (Cleveland, OH).

**Statistical analysis**

The proteasome activity of lymphoblast cells between each group was compared by Student t test. A \( P < 0.05 \) was considered statistically significant.

**Results**

**Reduced PTEN protein levels and increased proteasome activity is observed in lymphoblasts derived from PHTS patients**

To assess the level of PTEN expression in PHTS patients, we investigated PTEN protein expression through Western blot analysis. We identified significantly reduced PTEN protein levels in PHTS lymphoblasts when compared with PTEN*WT* controls (at ~40% of control protein, \( P < 0.01 \), see Fig. IA and B). To determine the mechanism of protein loss, we measured PTEN mRNA levels by qRT-PCR in the same samples. Only PTEN-R335X resulted in significantly diminished PTEN mRNA transcript compared with the PTEN-WT controls (at ~65% of WT1, and ~40% of WT2, respectively). The other 2 mutants, R233X and C136R,
showed PTEN mRNA levels similar to those of the controls (Fig. 1C). Our data suggest that posttranslational degradation is an important pathway for quality control of mutant PTEN protein in patients with PHTS.

We then assessed proteasome activity for 12 PHTS patients with germline PTEN nonsense or missense mutations (R233X, R335X, and C136R, n = 4 in each group) and 10 PTEN-WT controls. We found an approximately 60% and 30% increase in the proteasome activity of lymphoblasts isolated from PHTS patients with R233X or R335X nonsense mutations, respectively (Fig. 1D). We also found approximately 30% increase in proteasome activity in the C136R mutants. We therefore conclude that at least a subset of PHTS-related PTEN nonsense or missense germline mutations have diminished PTEN protein levels and proteasome hyperactivity.

**PHTS patients with robust proteasome activity are associated with neurological symptoms**

Because proteasome hyperactivity is associated with neurodegenerative diseases, and proteasome inhibition can alleviate such diseases, we hypothesize that patients with PHTS with increased proteasome activities may present with more neurologic phenotypes (14–16). To this end, we analyzed phenotypes in these 12 patients whose proteasome activities were elevated. Surprisingly, 4 of 12 (33.3%) proteasome hyperactive patients presented with such neurologic phenotypes as autism and mental retardation. In contrast, in another series of 39 patients harboring other PTEN germline mutations (G129Q, R130X, R130G, −903G>A) that did not significantly alter proteasome activity, only 3 patients (7.7%) had severe neurologic phenotypes (P = 0.04, Fisher 2-tailed exact test. Data not shown). Therefore, it is possible that patients with PTEN mutations resulting in unstable PTEN proteins and proteasome hyperactivity are more susceptible to develop neurologic phenotypes than patients with normal proteasome activities (Table 1).

**Missense mutations in PTEN affect protein stability**

Because our data clearly show increased proteasome activity in cells derived from patients with PHTS, it was necessary to investigate the effect of mutant PTEN protein on proteasome activity. We therefore transfected MCF-7 breast cancer cells with either WT or missense mutant PTEN (K62R, K125E, M3M4 or C136R) and investigated PTEN protein stability through a cycloheximide chase study. The WT and K125E mutant PTEN protein was stable after synthesis and was observed at 100% even after 8 hours of cycloheximide treatment. In contrast, PTEN-C136R

### Table 1. Patient genotypes, age at onset of symptoms and stability in lymphoblasts harboring PTEN germline mutations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age at onset (years)</th>
<th>Predicted size (KDa)b</th>
<th>DNA mutation</th>
<th>Amino acid change</th>
<th>Exon</th>
<th>Stability</th>
<th>Main manifestation</th>
</tr>
</thead>
<tbody>
<tr>
<td>900WMa</td>
<td>Male</td>
<td>5</td>
<td>55</td>
<td>406T→C</td>
<td>C136R</td>
<td>5</td>
<td>Unstable</td>
<td>Macrocephaly, Autism, Global developmental delay, Lipoma</td>
</tr>
<tr>
<td>622UC</td>
<td>Female</td>
<td>53</td>
<td>55</td>
<td>406T→C</td>
<td>C136R</td>
<td>5</td>
<td>Unstable</td>
<td>Ductal breast Ca., Thyroid adenoma, Thyroid Hurthle cell Ca., Uterine Ca., Lipoma</td>
</tr>
<tr>
<td>2447DY</td>
<td>Male</td>
<td>38</td>
<td>55</td>
<td>406T→C</td>
<td>C136R</td>
<td>5</td>
<td>Unstable</td>
<td>Macrocephaly, Thyroid adenoma, Hemangioma of skin</td>
</tr>
<tr>
<td>2447AY</td>
<td>Male</td>
<td>9</td>
<td>55</td>
<td>406T→C</td>
<td>C136R</td>
<td>5</td>
<td>Unstable</td>
<td>Macrocephaly, Hemangioma of skin</td>
</tr>
<tr>
<td>12961SW</td>
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<td>27</td>
<td>697C→T R233X</td>
<td>7</td>
<td>Unstable</td>
<td>NA</td>
<td></td>
<td>Macrocephaly, Mental retardation</td>
</tr>
<tr>
<td>972KSa</td>
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<td>7</td>
<td>27</td>
<td>697C→T R233X</td>
<td>7</td>
<td>Unstable</td>
<td>NA</td>
<td>Macrocephaly, Cafe-au-lait spots, Tan macules, Congenital genitourinary tract abnormalities</td>
</tr>
<tr>
<td>4026CA</td>
<td>NA NA</td>
<td>27</td>
<td>697C→T R233X</td>
<td>7</td>
<td>Unstable</td>
<td>NA</td>
<td></td>
<td>Macrocephaly, Breast cancer, Lipoma, Hodgkin’s lymphoma</td>
</tr>
<tr>
<td>420JT</td>
<td>Male</td>
<td>5</td>
<td>27</td>
<td>697C→T R233X</td>
<td>7</td>
<td>Unstable</td>
<td>NA</td>
<td>Macrocephaly, Thyroid adenoma, Renal cell carcinoma</td>
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<tr>
<td>531LMa</td>
<td>Male</td>
<td>8</td>
<td>39</td>
<td>1003C→T R335X</td>
<td>8</td>
<td>Unstable</td>
<td>Macrocephaly, Global developmental delay</td>
<td></td>
</tr>
<tr>
<td>1113KT</td>
<td>Female</td>
<td>33</td>
<td>39</td>
<td>1003C→T R335X</td>
<td>8</td>
<td>Unstable</td>
<td>Macrocephaly, Breast cancer, Lipoma, Hodgkin’s lymphoma</td>
<td></td>
</tr>
<tr>
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<td>Female</td>
<td>10</td>
<td>39</td>
<td>1003C→T R335X</td>
<td>8</td>
<td>Unstable</td>
<td>Macrocephaly, Autism, Cafe-au-lait spots, Thyroid adenoma</td>
<td></td>
</tr>
<tr>
<td>3147PD</td>
<td>Female</td>
<td>44</td>
<td>39</td>
<td>1003C→T R335X</td>
<td>8</td>
<td>Unstable</td>
<td>Macrocephaly, Thyroid adenoma, Renal cell carcinoma</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: NA, not available.

a Patients with severe neurologic phenotypes.
b Predicted size (KDa) was calculated online: http://web.expasy.org/compute_pi/
turned out to be highly unstable, as 50% of the mutant protein was degraded after 2 hours of cycloheximide treatment. Moreover, MCF-7 cells expressing PTEN-K62R or PTEN-M3M4 had PTEN levels decreased by 42% and 55%, respectively, after cycloheximide treatment for 8 hours. The data in Fig. 2A and B clearly show the varying stabilities of different mutant PTEN proteins. As some of the missense mutations dramatically impair PTEN’s protein stability, we next investigated whether degradation of the mutant PTEN was through the ubiquitin-proteasomal pathway. We first used HEK-293 cells, which were transiently transfected with FLAG-tagged WT or missense PTEN plasmids for 1 day and treated with 10 μmol/L MG132 or DMSO vehicle control for overnight. Cells were then harvested and probed for transfectant PTEN using anti-FLAG antibody and anti-GAPDH as a loading control. Arrow denotes PTEN that is cleaved by activated caspases. D, densitometric analyses of PTEN protein expression. PTEN protein levels were normalized against GAPDH and were presented as fold changes relative to control DMSO; bars, SD. *, *P < 0.05; **, **P < 0.01 when compared with the DMSO control. E and F, expression of missense mutant PTEN increases proteasome activity in vitro. MCF-7 and HEK-293 cells, respectively, expressing PTEN-WT or missense mutant PTEN were grown to subconfluence and whole-cell lysates were harvested using proteasome lysis buffer. The fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC was used to measure proteasome activity in whole-cell lysates. The proteasome activity was quantified and expressed relative to that of the MCF-7 or HEK-293 cells expressing the PTEN-WT control. Columns, mean from three experiments; bars, SD. *, *P < 0.05; **, **P < 0.01 versus PTEN-WT control. G, graphic representation of PTEN somatic and germline mutations in the proteasome study. The PTEN gene contains 9 exons and encodes a 403 amino-acid protein. The domains within PTEN include an N-terminal phosphatase domain and a C-terminal C2 domain. A PDZ- binding motif is also labeled in the C-terminus.
inhibitor, MG132 (10 μmol/L). Proteasomal inhibition results in an increase of the 3 unstable mutants, K62R, M3M4 and C136R, but not of the stable PTEN-mutant K125E and WT PTEN (Fig. 2C and D). We then repeated the same experiment in a breast cancer cell line, MCF-7. Similar to HEK-293 cells, inhibition of proteasome by MG-132 results in elevated PTEN protein levels only in cells expressing unstable mutants (K62R, M3M4, and C136R) (Supplementary Fig. S1). Our results suggest that certain missense PTEN mutants are degraded by the proteasomal pathway.

**Cells expressing missense PTEN mutants have elevated proteasome activity in vitro**

As we found different mutations leading to different PTEN protein stability, we next sought to determine whether cells expressing mutant PTEN with impaired protein stability would have elevated proteasome activity, as we already detected in PHTS-patient-derived lymphoblasts. Proteasome activity was measured in MCF-7 and HEK-293 cells expressing various PTEN mutations (Fig. 2E–G). Increased proteasome activity was detected in both MCF-7 and HEK-293 cell lines expressing PTEN-M3M4 and PTEN-C136R, which are unstable PTEN mutants. In contrast, cells expressing PTEN-K62R and PTEN-K125E showed similar proteasome activity with the WT control, although PTEN-K62R is also an unstable PTEN mutant (Fig. 2E and F).

**Subcellular distribution of proteasome activity in cells expressing PTEN variants**

Several mutations in PTEN have been shown to cause mislocalization (17–19). The relatively normal proteasome activity in cells expressing the unstable PTEN-K62R mutants prompted us to investigate proteasome activity at the subcellular levels. Using confocal immunofluorescence microscopy, we were able to define subcellular localization of the PTEN mutants in MCF-7 cells. Both PTEN-K62R and PTEN-K125E are predominantly nuclear localized, whereas PTEN-M3M4, PTEN-C136R, and WT are predominantly cytosolic (Fig. 3A).

![Figure 3. Subcellular distribution of proteasome activity in cells expressing PTEN variants. A, confocal microscope analyses of MCF-7 cells transfected with FLAG-tagged WT or missense mutant PTEN (M3M4, K62R, C136R, and K125E). The subcellular localization and stability of the analyzed PTEN mutants was labeled. B and C, MCF-7 (B) and HEK-293 (C) cells were fractionated. The cytosolic (left) and nuclear (right) fractions were analyzed for proteasome activity, respectively. Columns, mean from 3 experiments; bars, SD. * P < 0.05 versus WT, ** P < 0.01 versus WT control. The degree of fractionation is shown in Supplementary Fig. S2.](image-url)
As nuclear proteasome activity accounts for a minimal proportion of total activity (20), it is possible that a activity elicited by PTEN-K62R was too insignificant to create an observable change in the whole cell readout. To directly analyze proteasome activity in cytosolic and nuclear compartments, we performed subcellular fractionation and measured proteasome activity in both fractions. MCF-7 and HEK-293 cells overexpressing PTEN-M3M4 and PTEN-C136R showed significantly increased proteasome activity only in the cytosol but not in the nucleus. In contrast, overexpression of PTEN-K62R led to significantly increased proteasome activity only in the nucleus but not in the cytosol (Fig. 3B and C and Supplementary Fig. S2).

**Elevated proteasome activity in mice expressing mutant Pten**

To further show that PTEN-M3M4 is associated with increased proteasome activity in vivo, we measured the activity of the 20S proteasome in a Pten\(^{M3M4}\) knock-in murine model. Heterozygous (Pten\(^{WT/M3M4}\)) mice express the Pten-M3M4 protein monoallelically. In comparison with WT littermate controls, the 20S proteasome activity was increased by approximately 40% in the megencephalic brain tissues of the heterozygous mice (Fig 4A). These observations prompted us to further examine whether the ubiquitin levels were also upregulated in Pten\(^{WT/M3M4}\) heterozygous mice. Ubiquitin protein levels were compared in brain tissues pooled from two Pten\(^{WT/M3M4}\) heterozygous mice or from two Pten\(^{WT/WT}\) littermate controls. Strikingly, the megencephalic brains from the Pten\(^{WT/M3M4}\) heterozygous mice showed significantly increased ubiquitin abundance when compared with normoecephalic brains from WT controls (Fig. 4B, top). Decreased Pten levels, together with elevated ubiquitinated PTEN levels, were also confirmed in the brain tissues of the Pten\(^{WT/M3M4}\) heterozygous mice compared with those of the WT control litters (Fig. 4B, middle and Fig. 4C). Thus, these data confirm our hypothesis that proteasome activity can be elevated in MCF-7 cells, HEK-293 cells, and in a mouse model that express PTEN carrying specific mutations.

**Nonsense mutations in Pten affect protein stability and proteasome activity**

To study the effect of nonsense mutations on PTEN protein stability and proteasome activity, we introduced the 2 most common PHTS-related PTEN nonsense mutations (R233X and R335X) into MCF-7 and HEK-293 cells. Western blot data from both cell lines showed low to no observable PTEN-R233X and R335X proteins in contrast to PTEN-WT (Fig. 5A and B, lanes 3 and 5). We next asked whether proteasomal degradation also plays a role in the degradation of nonsense-mutant PTEN. We compared protein levels by Western blot analysis after transient transfection of FLAG-tagged PTEN-WT or PTEN-R233X or -R335X into MCF-7 and HEK293 cells, in the presence of proteasome inhibitor. Surprisingly, there was a significantly increased amount of truncated protein following proteasome inhibitor treatment in comparison with untreated cells (Fig. 5A and B, lanes 4 and 6). Thus, these 2 nonsense Pten mutants may undergo some proteasome degradation, at least in vitro.

To confirm our observations in PHTS, we analyzed proteasome activity in both cell lines to determine whether the instability was associated with proteasome hypersensitivity. Similar to our in vivo data, proteasome activity was inversely correlated with protein levels and was significantly elevated in comparison to the WT control (Fig. 5C and D). Such data further validates our hypothesis that proteasome hyperactivity is, in part, due to PTEN protein instability.

**Both the intact protein phosphatase activity and the steady-state of PTEN determine PTEN’s inhibitory effect on proteasome activity**

The activated proteasome in PHTS-derived cells and animal model prompted us to investigate the mechanism(s) by which PTEN mutations lead to proteasome hyperactivity. PTEN has both lipid and protein phosphatase activity.
Cancer Res; 73(10) May 15, 2013

Discussion

In the present study, we found increased proteasome activity in lymphoblast cells from PHTS patients and in cell lines expressing germ-line or somatic PTEN nonsense and missense mutations. The instability of both the nonsense and missense PTEN mutants can be rescued by blocking proteasome activity through a proteasome inhibitor, MG-132, whose clinical analog, bortezomib has already been used to treat patients with multiple myeloma (24) or patients with mantle cell lymphoma (25). In addition, our study revealed that certain PTEN missense or nonsense mutations that affect proper PTEN folding and/or protein phosphatase activity are associated with elevated proteasome activity in PHTS patient-derived cells and in breast cancer cells. Taken together, we propose that there are 2 mechanisms for gain-of-function proteasomic activity by PTEN mutations.

PTEN mutations that impair the protein phosphatase activity will effect enhanced proteasome activity through a proteasome inhibitor, MG-132, whose clinical analog, bortezomib has already been used to treat patients with multiple myeloma (24) or patients with mantle cell lymphoma (25). In addition, our study revealed that certain PTEN missense or nonsense mutations that affect proper PTEN folding and/or protein phosphatase activity are associated with elevated proteasome activity in PHTS patient-derived cells and in breast cancer cells. Taken together, we propose that there are 2 mechanisms for gain-of-function proteasomic activity by PTEN mutations. PTEN mutations that impair the protein phosphatase activity will effect enhanced proteasome activity through uncontrolled MAPK/ERK pathway, whereas PTEN mutations that impair PTEN protein stability will activate proteotoxic stress and induce proteasome activity. Those 2 effects can be
Figure 6. Both intact protein phosphatase activity and the steady-state of PTEN determine PTEN’s inhibitory effect on proteasome activity. A, expression of PTEN suppresses proteasome activity and inhibits the PI3K/AKT and MAPK pathways. MCF-7 cells were transfected with PTEN or the empty vector. Cells were harvested after 24 hours for proteasome activity (left) and Western blot analysis (right). B, inhibition of the AKT pathway does not significantly alter proteasome activity. MCF-7 cells were treated with the AKT inhibitor, perifosine (0, 5, and 10 μmol/L), for 24 hours. Cells were then harvested for proteasome activity assay (left) and Western blot analysis (right). C, expression of constitutively active AKT1 (MyrAKT) does not alter proteasome activity. Proteasome activity of MCF-7 cells expressing MyrAKT or a control vector (left) for 24 hours. Western blot analysis for basal levels of AKT and P-Akt in MCF-7 cells expressing MyrAKT or a control vector (right). D, inhibition of the MAPK pathway significantly decreases proteasome activity. MCF-7 cells were treated with a mitogen-activated protein kinase (MAPK) inhibitor, PD98059 (0, 5, and 10 μmol/L), for 24 hours. Cells were then harvested for proteasome activity assay (left) and Western blot analysis (right). E, PTEN mutations lead to changes in PTEN stability and the PI3K/AKT, MAPK/ERK signaling pathways. MCF-7 cells were transiently transfected with FLAG-tagged WT or mutant PTEN (left). Cells were then harvested after 24 hours for FLAG-PTEN, P-AKT, and P-ERK by Western blotting. Normalization of FLAG-PTEN, P-AKT, and P-ERK of cells expressing mutant PTEN to those of cells expressing PTEN-WT (right). The expression of each protein was normalized to the GAPDH control, and the relative protein levels were normalized to those of the WT control. F, model of the mechanisms through which mutant PTEN enhances proteasome activity. PTEN mutations that impair the protein phosphatase activity of PTEN will have enhanced proteasome activity through uncontrolled upregulation of the MAPK/ERK pathway, whereas PTEN mutations that impair the stability will activate the proteotoxic stress pathway and induce proteasome activity. Those 2 effects can be additive if mutations have both defects.
additive in mutations that have both defects. The detailed mechanisms have been illustrated in Fig. 6F.

PTEN is a 403-amino acid protein with 2 major functional domains: an N-terminal domain encompassing exons 1–6 and a C-terminal domain encompassing exons 6–9 (26). According to The Human Gene Mutation Database (www.hgmd.cf.ac.uk/), approximately 50% of PTEN mutations are missense or nonsense mutations. Thus, understanding the pathogenic effects of PTEN missense/nonsense mutations will benefit patients who harbor such mutations. Here, we show that 3 missense mutations (K62R, M3M4, C136R) and 2 common nonsense mutations (R233X, R335X) exhibit enhanced proteosomal degradation of PTEN providing a mechanism for their impaired protein stability. Such quantitative loss of PTEN results in the functional insufficiency of protein function. Compared with missense PTEN mutations, the 2 common nonsense mutations are highly unstable, as PTEN protein cannot be detected if proteosomal activity is not inhibited. This may suggest the proteosomal degradation of missense mutant PTEN is incomplete whereas the degradation of nonsense mutant PTEN is complete. An alternative explanation might be that the missense mutants studied here are subject to proteosomal degradation secondary to misfolding, whereas these 2 common nonsense mutations result in protein, which undergoes both nonsense-mediated decay (C. Eng, unpublished observations) and proteosomal degradation.

We additionally show that PHTS patients with certain PTEN nonsense or missense mutations have highly activated proteasomes in their derived lymphoblast cells. Moreover, MCF-7 breast cancer cells and HEK-293 cells expressing these missense mutant PTEN (PTEN-M3M4, C136R) and nonsense mutant PTEN (PTEN-R233X and PTEN-R335X) proteins, all showed increased proteasome activity compared to the same cell lines expressing the PTEN-WT control. We believe that our results here identify a potential intrinsic link between presumably misfolded mutant PTEN and the elevated proteasome activity. Interestingly, patients with PHTS with destabilizing PTEN mutations and proteasome hyperactivity are more susceptible to develop neurologic symptoms such as mental retardation and autism than patients with normal proteasome activities. In addition to PHTS, PTEN is mutated in patients with autism spectrum disorders (ASD). Rodríguez-Escudero and colleagues identified distinctive functional patterns among PTEN mutations found in tumors and in the germline of patients with PHTS and ASD. They revealed that ASD-associated hereditary PTEN mutations did not substantially abrogate PTEN activity in vivo, whereas most of PHTS-associated mutations did (27). By studying neurologic symptoms in patients with PHTS, our results do suggest that PTEN stability and subsequent proteasome activity may be another factor that influences neurologic phenotypes in PHTS.

Missense mutations may affect not only protein instability but intracellular localization. Our studies have revealed that localization of mutant PTEN contributes to total and subcellular proteasome activity. PTEN is predominantly localized in the cytosol. Cells overexpressing the cytosolic-localized mutant PTEN-M3M4 and PTEN-C136R have very robust proteasome activity in whole-cell lysates and the cytosolic compartment when compared to cells overexpressing other PTEN mutants or WT PTEN. In contrast, K62R and K125E, which are nuclear mislocalized PTEN mutants, have very different patterns of proteasome activity. The PTEN-K125E protein, although nuclear mislocalized, is relatively stable consistent with the accompanying normal proteasome activity, suggesting that it may not be a major substrate of the ubiquitin-proteasome system. To our surprise, the K62R-mutant protein, which is both unstable and nuclear mislocalized, shows relatively normal proteasome activity in whole-cell lysates but elevated nuclear proteasome activity when compared with the WT control cells and cells expressing other PTEN mutants. Therefore, mislocalized mutant PTEN seems to only induce proteasome activity in the compartment where it is degraded. Nuclear predominant mutant PTEN (e.g., K62R) induces nuclear proteasome activity, whereas cytosolic predominant mutant PTEN (e.g., PTEN-M3M4) induces cytosolic proteasome activity. Indeed, the nuclear ubiquitin-proteasome system is responsible for the proteasomal degradation of many short-lived nuclear proteins (28). For instance, MyoD, which is a nuclear transcription factor that is pivotal in skeletal muscle differentiation, is degraded by the nuclear proteasome system (29). Increased nuclear proteasome activity is associated with stress and drug resistance in solid tumors (30). Further studies are needed to elucidate the specific mechanism and pathogenesis of the proteasome hyperactivity induced by nuclear mislocalized mutant PTEN-K62R.

One major goal of this study was to elucidate the mechanisms of proteasome activation in PTEN mutant cells. A detailed molecular and functional analysis shows that the inhibition of MAPK activation by PD98059 led to suppressed proteasome activity, whereas no effect was observed with inhibition of the AKT activation by perifosine. (Fig. 6). We revealed that both PTEN-WT overexpression and PD98059 treatment only suppressed proteasome activity by approximately 20% in MCF-7 (Fig. 6A), whereas in the same cell line expressing unstable PTEN mutants (such as PTEN-M3M4 and PTEN-C136R; see Fig. 2E), the latter induced more than 50% increase of proteasome activity when compared with cells expressing PTEN-WT. Therefore, it is plausible to conclude that PTEN mutants most likely cause proteasome hyperactivity via 2 different mechanisms, namely, induction of proteotoxic stress and loss of protein phosphatase activity.

The proper turnover of the mutant PTEN protein by proteasomes may have essential meaning. In both human and mouse model, PTEN dose predisposes to cancer susceptibility (7, 31). Patients with PTEN mutations that encode unstable proteins will result in PTEN dosage effects, whereas patients with PTEN mutations that encode stable proteins may result in a dominant-negative state. In addition, some mutations that abolish PTEN’s phosphatase activity may still have PTEN’s tumor suppressor effects (32). Truncated PTEN can also change the binding ability to microRNAs that affect PTEN protein levels (33).

Proteasome hypersensitivity may be a hallmark of human cancers. Chen and colleagues reported that the activity of proteasomes was increased in more than 90% of primary
breast cancer tissue specimens. In contrast, no activation was observed in benign solid tumors (11). Another study revealed increased proteasome subunit protein expression and proteasome activity in colon cancer (34). In cancer cells, intrinsic stress response pathways are frequently activated. Accumulation of misfolded PTEN protein may lead to proteotoxic stress (35). This can lead to progressive cellular dysfunction such as activation of prosurvival pathways and proliferation pathways (such as heat shock factors). In recent years, proteasome inhibitors have been increasingly developed and tested in human cancers. Thus, our study has some impact on clinical practice by underscoring proteasome inhibitors in the treatment of breast and other cancers with proteasome-related PTEN instability. Indeed, 2 proteasome inhibitors (bortezomib and BU-32) have been tested to be effective in cultured breast cancer cells and in breast cancer xenografts (36, 37).

In conclusion, we identified that proteasome hyperactivity and proteotoxic stress may be a common phenomenon in patients with PHTS with different PTEN nonsense or missense mutations, at least in certain subsets. Our observations here may also explain why there only exists loose genotype-phenotype correlation in PHTS: seemingly disparate types and locations of mutations result in proteins with common fates, in this situation, protein instability and proteasome hyperactivity. We also found that relative proteasome hyperactivity can be affected by PTEN protein stability, protein phosphatase activity, and subcellular localization. These data contribute to a better understanding that PTEN nonsense and missense mutations have multiple deleterious effects, and the combination of P38K pathway inhibitors and agents targeting proteasomes may show promise for prevention or treatment of breast tumors in a subset of such mutation carriers or in sporadic malignancies showing similar PTEN protein dysfunctional end points.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


