Translational Oncology
www.transonc.com

Volume 7 Number 2 April 2014 pp. 196–205.e1 196

# Combined PTEN Mutation and Protein Expression Associate with Overall and Disease-Free Survival of Glioblastoma Patients[1,2]

**Jie Xu[*,3], Zhaoli Li[†,3], Jilin Wang[‡], Haoyan Chen[*,§] and Jing-Yuan Fang[*,¶]**

*State Key Laboratory for Oncogenes and Related Genes, Division of Gastroenterology and Hepatology, Renji Hospital, Shanghai, China; [†]Viral Genetics Laboratory, Vrije Universiteit Brussel, Brussels, Belgium; [‡]Shanghai Institute of Digestive Disease, Renji Hospital, Shanghai, China; [§]Shanghai Jiao Tong University School of Medicine, Renji Hospital, Shanghai, China; [¶]Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Shanghai, China

## Abstract

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor commonly inactivated in glioblastoma multiforme (GBM), but the prognostic significance of PTEN remains controversial. Here, we demon- strate significant prognostic value of combined PTEN mutation and expression for the survival of patients with GBM on the basis of analysis of large-scale cancer genomic data. PTEN nonsense mutations associated with sig- nificantly shorter disease-free survival and overexpression of PTEN protein linked to shorter disease-free and overall survival of patients with GBM. PTEN nonsense mutations correlated with decreased p53 and Gata3 protein levels and increased genomic instability in human GBM tissues. Expression of nonsense PTEN mutant decreased p53 and Gata3 levels, producing increased DNA damage both in vitro and in vivo. Mice carrying xenograft tumors with nonsense PTEN mutant displayed significantly shorter survival. Our data demonstrated the prognostic value of combined PTEN mutation and protein expression for patients with GBM and highlighted distinct biologic effects of nonsense and missense mutations of PTEN.

*Translational Oncology (2014) 7, 196–205.e1*

## Introduction

Glioblastoma multiforme (GBM) is the most common malignant brain tumor and one of the most aggressive human cancers, with a mean survival time of less than 1 year after diagnosis [1]. Loss of 10q, including phosphatase and tensin homolog deleted on chromosome 10 (PTEN ) gene, is the most common alteration associated with GBM (70% incidence) [2]. PTEN is a tumor suppressor that acts as a phosphatase for the lipid signaling intermediate phosphatidylinositol-3,4,5-trisphosphate (PIP3), producing phosphatidylinositol-4,5- bisphosphate. PIP3 anchors AKT to the membrane, where AKT is activated through its phosphorylation by phosphoinositide-dependent kinase-1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2). AKT phosphorylates numerous targets to transduce sig- nals for growth, proliferation, and survival [3]. In addition to its effect on PIP3/AKT pathway, PTEN also regulates p53 function. Mouse double minute 2 homolog (MDM2) is a substrate of AKT, thus acti- vation of AKT on PTEN loss results in MDM2 phosphorylation and

Address all correspondence to: Jie Xu, MD, or Haoyan Chen, MD, or Jing-Yuan Fang, MD, State Key Laboratory for Oncogenes and Related Genes, Division of Gastroenterology, Shanghai Institute of Digestive Disease, Shanghai Jiao Tong University School of Medicine Renji Hospital, Shanghai, China. E-mail: xujieletter@gmail.com

[1] This project was supported by grants from the National Basic Research Program of China (973 Program; 2010CB5293), the National High Technology Research and Development Program of China (863 Program; 2012AA02A504), the Program for Innovative Research Team of Shanghai Municipal Education Commission, and National Natural Science Foundation of China (30971330, 31371420, and 81320108024) to J.Y.F. The project was also partially supported by National Natural Science Foundation of China (81000861, 81322036, and 81272383), Shanghai "Oriental Scholars" project (2013XJ), Shanghai Science and Technology Commission "Pujiang Project" (13PJ1405900), and Shanghai Natural Science Foundation (12ZR1417900) to J.X. The sponsors of this study had no role in the collection of the data, the analysis and interpretation of the data, the decision to submit the manuscript for publication, or the writing of the manuscript. The authors have no conflict of interest.

[2] This article refers to supplementary material, which is designated by Figure W1 and is available online at www.transonc.com.

[3] These authors contributed equally to this work.

Received 18 September 2013; Revised 18 September 2013; Accepted 22 November 2013

Copyright © 2014 Neoplasia Press, Inc. All rights reserved 1936-5233/14
http://dx.doi.org/10.1016/j.tranon.2014.02.004

increased nuclear import to enhance p53 degradation [4]. PTEN also physically associates with p53 to enhance its DNA binding ability [5]. The domains within PTEN include a phosphatidylinositol-4, 5-bisphosphate–binding region, a phosphatase domain, a C2 domain, with a C-terminal tail containing two rich in proline, glutamic acid, serine, and threonine (PEST) domains for degradation and a post synaptic density (PDZ) interaction motif (Figure 1*A*). Mutations of PTEN in GBM include missense, nonsense, frameshift, and splice site mutations distributed throughout the gene, causing disruption of the phosphatase domain by truncation or instability. The most frequently observed mutations in central nervous system (CNS) tumors are amino acid substitutions at arginine 173 and nonsense mutation at arginine 130. The preferential selection of these "hot spots" suggests that mutants of PTEN may not confer equal oncogenic effects in GBM [6].

The prognostic significance of PTEN in GBM is still a matter of debate. Although multiple clinical studies have suggested that PTEN mutation in glioma has no correlation with survival or chemosensitivity [7–10], some other studies have associated loss of function of PTEN with a more adverse outcome [11–13]. Unfortunately, many of these studies lack the sample size or thorough evaluation of PTEN genetic alterations to make concrete conclusions. To precisely evaluate the genuine prognostic significance of PTEN function in brain malig- nancies, comprehensive analysis of GBM at the genetic and expression levels on a large number of morphologically well-defined patients is required [14].

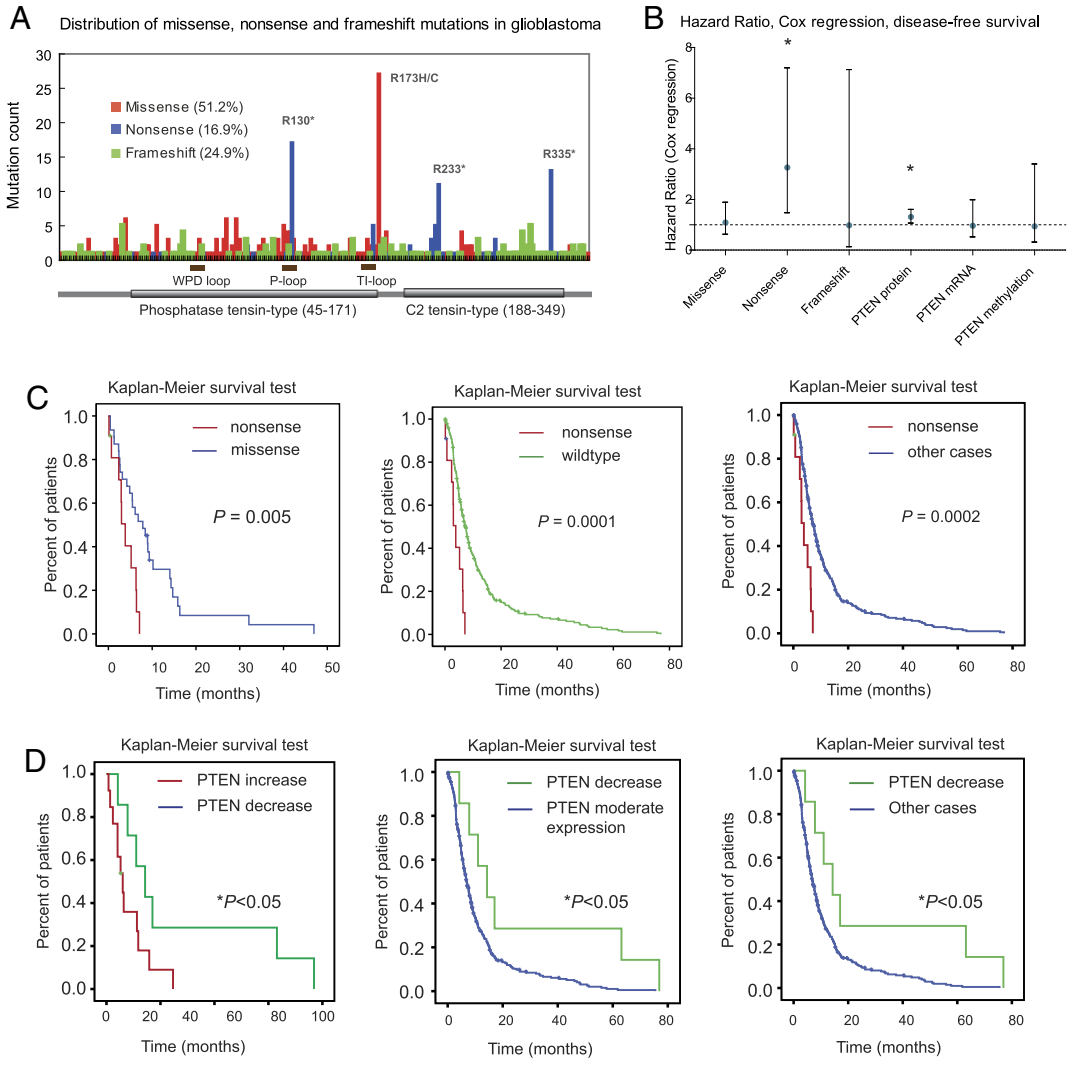


**Figure 1.** Distinct effects of PTEN mutations on DFS of patients with glioblastoma. (A) The mutation spectrum of PTEN gene in the TCGA GBM database. Missense, nonsense, and frameshift mutations are respectively plotted in red, blue, and green. The nonsense mutations on position $Arg^{130}$ and missense mutations on $Arg^{173}$ represent the most frequent mutations in GBM, which are in close relation with the phosphatase tensin–type domain. (B) Effects of PTEN mutation, expression, and promoter methylation on DFS of patients with GBM. The effects of different factors on DFS time were analyzed using Cox regression, and the HR for each indicated factor is plotted (round dot) with 95% CIs (upper and lower bars). Nonsense PTEN mutation and increased Pten protein level are independent factors that correlate with shorter DFS ($P < .05$, star signs). (C) Kaplan-Meier survival tests suggest significantly shorter DFS for patients with GBM carrying nonsense mutations than those carrying missense mutations (left panel) or wild-type PTEN (middle panel). Nonsense mutations of PTEN also display significantly shorter DFS when compared to all other cases (right panel). The corresponding P values are indicated in each panel. (D) GBM cases overexpressing Pten protein display shorter DFS than other cases, as indicated by Kaplan-Meier survival test ($P < .05$, star signs).

In the present study, we perform a comprehensive analysis on the prognostic value of PTEN status in patients with GBM on the basis of large-scale cancer genomic data. The 586 GBM cases included in this study were well defined in both clinicopathologic and genomic/proteomic aspects and thus may add an important answer to this controversial field. We also analyze the effects of PTEN mutations on different signaling proteins and experimentally validated the results. By these efforts, we aim to provide mechanistic explanations for the distinct effects of PTEN mutations.

## Materials and Methods

### *Plasmid Construction*

The vectors expressing wild-type PTEN were cloned by inserting cDNAs into pcDNA3 vectors through the NheI and XhoI restriction sites. All mutant vectors were generated by site-directed mutagenesis polymerase chain reactions (Roche, Basel, Switzerland).

### *Cell Culture and Western Blot Analysis*

The Ishikawa cells were purchased from the American Type Culture Collection (Manassas, VA) and were passaged in our laboratory for less than 6 months. Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with glutamine, pyruvate, antibiotics, and 10% fetal calf serum in a humidified atmosphere containing 5% CO2 at 37°C.

Cell lysate proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% gels) and transferred to nitro- cellulose membranes. Protein amounts were quantified using the Bradford method, and equal protein amounts were loaded to the gel. Membranes were blocked in TBS with 0.05% Tween 20 (TBST) containing 5% nonfat dry milk powder for 1 hour. Western blots were probed with primary antibodies for 1 hour, washed three times with TBST, and then incubated with the appropriate secondary antibodies for 30 minutes. Membranes were then washed with TBST three times before developing with SuperSignal West Dura chemiluminescent substrate (Pierce, Rockford, IL).

### *The Comet Assay for Measuring DNA Damage*

The comet assay used to measure DNA damage has been described previously [15]. Briefly, cells were treated with 20 μM etoposide (Sigma, St Louis, MO) for 4 hours, and the damage distribution was measured as tail moment (product of tail length and fraction of DNA). Cells were harvested and resuspended in Hank's Balanced Salt Solution (Sigma) with 10% DMSO and 0.5 M EDTA. The cell suspension was then suspended in 0.7% low-melting agarose at 37°C (Sigma) and layered on to comet slides (Trevigen, Gaithersburg, MD). The cells were then lysed in lysis solution containing 2.5 M NaCl, 100 mM pH 8.0 EDTA, 10 mM Tris-HCl, 1% Triton X (Sigma) at 4°C for 1 hour.

Denaturation was carried out for 40 minutes, in chilled alkaline elec- trophoresis buffer (pH 13.0-13.7). Electrophoresis was subsequently carried out for 20 minutes. Slides were immersed in neutralization buffer (500 mM Tris-HCl, pH 7.4), dehydrated, dried and stained with SYBR Green dye (Invitrogen, Carlsbad, CA), and scored with OpenComet plugin of ImageJ software. The images were captured using fluorescence microscope (Carl Zeiss, Oberkochen, Germany) equipped with triple-band filter. Fifty comets per sample were randomly selected and analyzed. The extent of DNA damage was expressed as tail moment, which corresponded to the fraction of the DNA in the tail of the comet.

### *In Vivo Experiments*

Briefly, male BALB/c athymic nude mice (4-5 weeks old) were obtained from the Experimental Animal Center of Shanghai Institutes for Biological Sciences (Shanghai, China). Mice were randomly divided into the following two groups: nonsense group and missense group (15 mice per group). Nonsense-group mice were injected subcutaneously into the right flank with $1.0 \times 10^7$ Ishikawa cells stably transfected with PTEN nonsense mutant (R130*), whereas the missense-group mice were injected with $1.0 \times 10^7$ Ishikawa cells bearing PTEN missense mutant (R173H) to establish xenograft models. Tumor diameters were measured with digital calipers, and tumor volume was calculated by the following formula: tumor volume ($mm^3$) = shorter diameter$^2$ × longer diameter/2. The tumor volume data are pre- sented as means ± SD (n = 15). Our study was approved by the Animal Care and Use Committee of the Renji Hospital affiliated to Shanghai Jiao Tong University School of Medicine. All animal procedures were performed according to the guidelines developed by the China Council on Animal Care and the protocol approved by the Renji Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

### *Cancer Genomic and Clinical Data*

The genomic sequencing and survival data analyzed in this study were obtained from The Cancer Genome Atlas (TCGA) GBM data set [16]. The published versions of these data sets include 586 cancer cases with sequencing data and clinical information.

### *Reverse Phase Protein Array Data*

The corresponding reverse phase protein array (RPPA) data of TCGA GBM data set were obtained using the cBioPortal online data portal (Memorial Sloan-Kettering Cancer Center, New York, NY) [17], which include quantified expression of 122 proteins and 43 phosphoproteins involved in various signaling pathways. Exam- ples of such pathways include p53 signaling, retinoblastoma (RB), AMP-activated protein kinase (AMPK), PTEN, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB), receptor tyrosine kinase (RTK)/RAS GTPase, and epidermal growth factor receptor signaling, and other sequences. A complete list of antibodies in the protein micro- array can be accessed from TCGA data portal (http://tcga-data.nci.nih. gov/tcga/, Memorial Sloan-Kettering Cancer Center). The patients with upper or lower quarter Pten protein expression were determined according to the levels detected by RPPA (respectively ranked as 25% highest or 25% lowest).

### *Drug Sensitivity Profile Data*

We obtained the sensitivity profiles of 59 human brain tumor cell lines to 131 anticancer drugs from the Cancer Cell Line Encyclopedia (CCLE; Broad Institute, Cambridge, MA) database [18]. The half-maximal inhibitory concentration (IC50) was used as a measure of the effectiveness of a drug on the cell lines. The mutation spectrum of TP53 in these cell lines was similar with that in the TCGA data sets.

### *Survival Analysis*

Survival analysis was carried out in R program using the "survival" package as described previously [19]. In the Kaplan-Meier (log-rank) survival test and Cox regression models, the censored status is indicated when the patient was still alive (or cancer free) at the time of follow-up. The Cox regression model included cancer type as a covariant, and the P value for mutation type is calculated after

adjustment for the factor of cancer type. The hazard ratios (HRs) and 95% confidence intervals (CIs) were also determined for each mutation. The effects of different p53 mutations were compared to nonsense mutations as an indication of gain-of-function (GOF) effect.

### *Clustering Analysis*

The GBM cases with either increased or decreased PTEN protein level were clustered according to their patterns of signaling protein expression (on the basis of RPPA data). The hierarchical clustering was carried out using the Cluster program (Pearson correlation, average linkage) and visualized by TreeView (University of California at Berkeley, CA) [20]. The functional profiling of each protein cluster was performed using the g:Profiler annotation tool (University of Tartu, Estonia) under the criteria of $P < .05$.

## Results

### *PTEN Nonsense Mutations Shorten Disease-Free Survival of GBM Patients*

We started by analyzing the effects of different PTEN mutations on disease-free survival (DFS) of patients with GBM, which reflects the effectiveness of treatment and the tendency for cancer recurrence. A total number of 586 patients with complete genomic sequencing and clinical data from the TCGA GBM data set [16] were selected for analysis in this study. The spectrum of PTEN mutation in the TCGA GBM data set was similar to that reported previously [14], including missense (51.2%), nonsense (16.9%), frameshift (24.9), and other types of mutations (7%; Figure 1*A*). Using Cox propor- tional hazards analysis, we analyzed the independent effects of PTEN mutation, promoter methylation and expression (protein and mRNA levels by arrays), genomic instability, and Karnofsky performance scale on DFS of patients with GBM. Intriguingly, nonsense muta- tions of PTEN associated with significantly shorter DFS (median, 3.8 months) than other mutations or wild-type genotype (median, 7.2 months), displaying higher HR of 3.26 (95% CI = 1.48-7.20; Figure 1, *B* and *C*). On the contrary, missense or frameshift muta- tions showed no significant association with DFS of patients with GBM. Moreover, overexpression of PTEN protein also associated with shorter DFS (median, 6.0 months) than other cases (median, 7.0 months), with increased HR of 1.31 (95% CI = 1.07-1.61; Figure 1, *B* and *D*). No correlation was found between patient DFS and PTEN mRNA level or promoter methylation, the number of mutations (as revealed by genomic sequencing), or fraction of genome with copy number alteration (CNA, an indication of genomic instability) in GBM cases.

### *PTEN Nonsense Mutations Increase Genomic Instability*

The different effects of PTEN mutations on DFS suggest that these mutations also confer distinct biologic consequences. Because loss of PTEN function has been linked to genomic instability and impaired DNA repair ability [21], we compared the number of mutations and fraction of genome with CNA in patients with GBM carrying different types of PTEN mutations. Although missense, non- sense, and frameshift mutations were all found to increase the fraction of genome with CNA (Mann-Whitney test, $P < .05$; Figure 2*A*), only nonsense mutation of PTEN associated with a higher number of mutations in GBM tissues (Mann-Whitney test, $P < .05$; Figure 2*B*). Furthermore, nonsense mutation of PTEN also linked to decreased levels of p53 and Gata3 proteins (Mann-Whitney test, $P < .05$), but such link was not evident for missense or frameshift mutations (Figure 2, *D* and *E*).

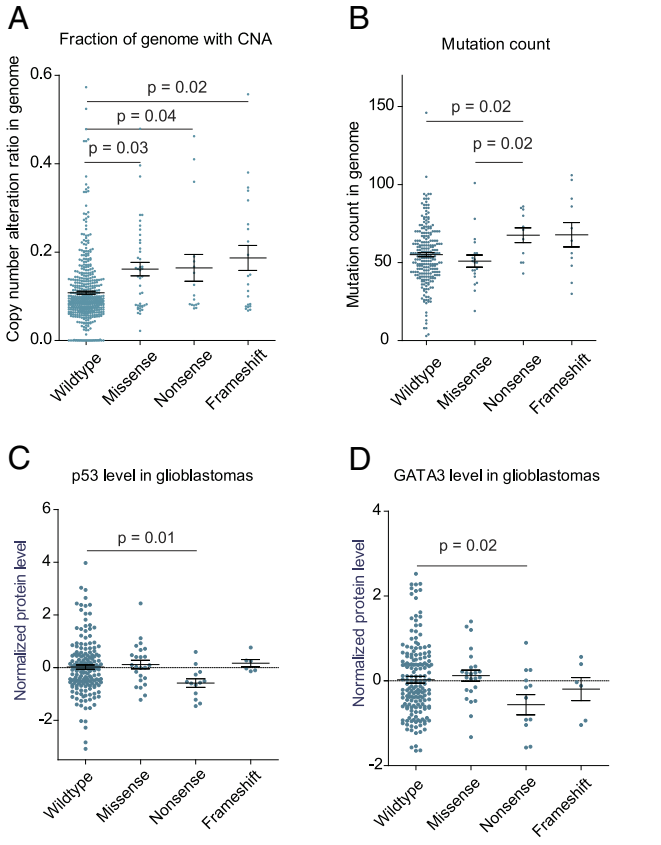


**Figure 2.** PTEN nonsense mutations increase genomic instability. (A) Fraction of genome with CNA of patients with GBM carrying different PTEN mutations. Each blue dot represents the CNA fraction (percentage value) of one case, and the average values were compared between patients carrying wild-type PTEN or missense, nonsense, or frameshift mutations using Mann-Whitney test. Missense and nonsense mutations showed significantly increased CNA fraction compared to the wild type ($P < .05$). (B) Nonsense PTEN mutations asso- ciate with increased number of mutations in the genomes of patients with GBM. The number of mutations in the genomes of patients with GBM was compared between wild-type, missense, nonsense, and frameshift groups using Mann-Whitney test. (C and D) Nonsense PTEN mutations associate with significantly lower levels of p53 and Gata3 proteins. The protein levels of p53 or Gata3 (as quantified by RPPAs) were compared between GBM cases with different PTEN genotypes (wild-type, missense, nonsense, or frameshift mutations) using Mann-Whitney test.

Validation of PTEN Mutational Effects In Vitro and In Vivo To test the different effects of PTEN mutations, we stably trans- fected "hotspot" PTEN nonsense (R130*) and missense (R173H) mutants to the human Ishikawa cell strain. The Ishikawa strain has a PTEN-null background [22], which facilitates the analysis on the effects of exogenous mutants. We performed the comet assay to test whether PTEN mutations could affect cell ability to repair DNA damage. As a result, the nonsense mutation conferred significantly higher extent of DNA damage when compared to the missense mutation (Figure 3, *A* and *B*), thereby confirmed the findings in patients with GBM. Furthermore, we validated the effects of PTEN mutations on p53 and Gata3 protein levels in Ishikawa cells using Western blot analysis. As expected, the nonsense mutation of PTEN completely lost the wild-type ability to increase p53 and Gata3 levels, but the missense

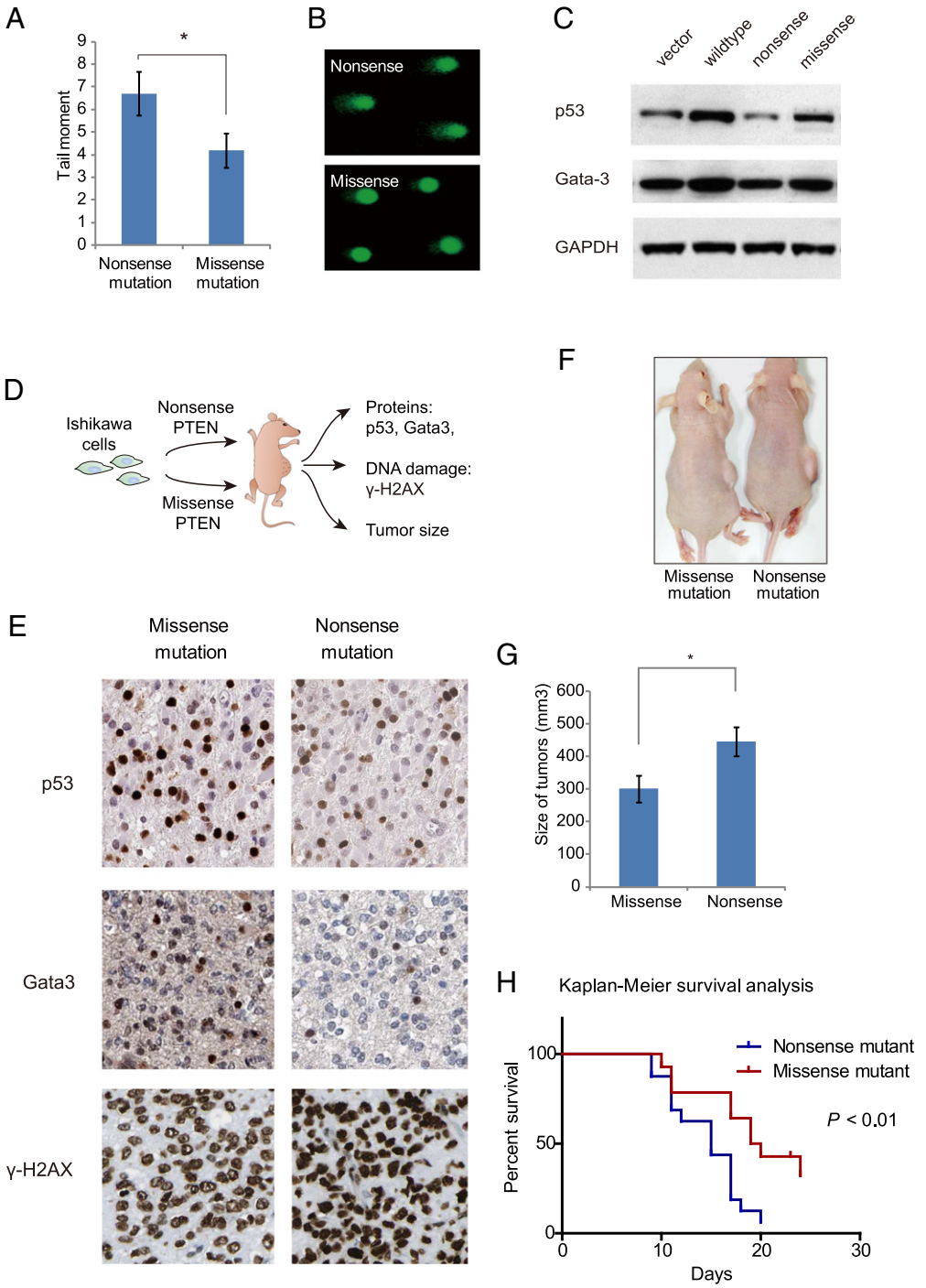


**Figure 3.** Validation of PTEN mutational effects in vitro and in vivo. (A) Induction of DNA damage in etoposide -treated cells expressing different PTEN mutants. The Ishikawa cells (PTEN null) were transfected with PTEN nonsense (R130*) or missense (R173H) mutants, followed by treatment with 20 $\mu$M etoposide for 4 hours. The damage distribution was measured as tail moment. The bar plots indicate the statistics of tail moments (means ± SE; *$P$ < .05, t test). (B) Representative images of SYBR Green–stained comets. (C) Effects of PTEN mutants on p53 and Gata3 levels as determined by Western blot analysis. The Ishikawa cells expressing nonsense (R130*) or missense (R173H) mutations were lysed and subjected to Western blot analysisfor the detection of p53 and Gata3 protein levels. The wild-type PTEN significantly increased p53 and Gata3 levels, but this ability was not found for the nonsense mutant. The missense mutant only mildly increased p53 and Gata3 levels. Full, uncut gels are shown in Figure W1. (D) Schematic representation of in vivo experimental procedures. Ishikawa cells stably expressing nonsense (R130*) or missense (R173H) mutant PTEN were subcutaneously injected into nude mice (n = 15 for each group). After formation of xenograft tumors, tissues were subjected to analyses as indicated. (E) Expression levels of p53, Gata3, and γ-H2AX in xenograft tumor tissues as determined by immunohistochemistry (IHC) assay. The left panels show representative IHC images from tumors carrying missense mutant, whereas the right panels include IHC images for nonsense mutant. Nonsense PTEN mutation associated with decreased level of p53 and Gata3 and increased level of γ-H2AX (a marker for increased DNA damage). (F) Representative images for mice carrying xenograft tumors that expressed nonsense mutant (larger, mouse on the right) or missense PTEN mutant (on the left). (G) Statistics of tumor sizes for two experimental groups (means ± SE; *$P$ < .05, t test). (H) Kaplan-Meier survival analysis shows shorter survival of mice carrying nonsense PTEN mutation (median survival, 15.0 vs 19.5 days, $P$ < .01).

mutation still retained residue activity (Figure 3*C* , full gel images in Figure W1). These results suggest stronger loss-of- function (LOF) effect displayed by nonsense mutations when com- pared to missense mutations. Gata3 has been shown to antagonize cancer progression in PTEN-deficient tumors, and this may also help to explain the stronger adverse effect of nonsense mutations on DFS. To provide experimental evidence for the different effects of PTEN mutations in vivo, we established mouse xenograft models by im- planting stable Ishikawa lines that express either nonsense (R130*) or missense (R173H) PTEN mutations to nude mice (experimental pro- cedures illustrated in Figure 3*D*). As expected, xenograft tumor tissues bearing the nonsense PTEN mutation displayed lower levels of p53 and Gata3 proteins (Figure 3*E*). Because γ-H2AX is a molecular marker for tumor genomic instability [23], we detected the level of H2A histone family, member X (γ-H2AX) in different xenograft tissues to validate the findings in patients with GBM. As shown in Figure 3*E*, tumors bearing the nonsense PTEN mutation expressed higher level of γ-H2AX, indicating greater genomic instability in these tumors. In addition, the presence of nonsense PTEN mutation also resulted in larger xenograft tumor size (Figure 3, *F* and *G* ) and shorter survival time (Figure 3*H*). Taken together, these results suggest that PTEN nonsense mutations contribute to tumor aggressiveness by increasing genomic instability and confirmed the findings in patients with GBM.

**Table 1.** Brief Description on the Activity and Effective Pathways of Bosutinib, Bryostatin, AZ628, and PAC-1.

| Drug Name | Activity | Pathway Linked to PTEN |
|---|---|---|
| Bosutinib | Src/Abl tyrosine kinase inhibitor | Src reverses wild-type PTEN function and inhibits AKT. |
| Bryostatin | PKC inhibitor | Loss of PTEN activates PKC. |
| AZ628 | Raf inhibitor | Loss of PTEN cooperates with RAS/MAPK activation. |
| PAC-1 | Activates procaspase 3 | Loss of PTEN inhibits caspase 3. |

### *Nonsense Mutations Display Characteristic Drug Sensitivity Profile*

To test whether PTEN nonsense mutations affect pharmacological responses, we analyzed CCLE that includes the sensitivity profiles of 59 human brain tumor cell lines to 131 anticancer drugs [18].

The sensitivity to each drug (IC50) was compared between cell lines carrying PTEN nonsense mutations or other mutations using Mann-Whitney test. Interestingly, PTEN nonsense mutations displayed lower sensitivity to the drug bosutinib [a proto-oncogene

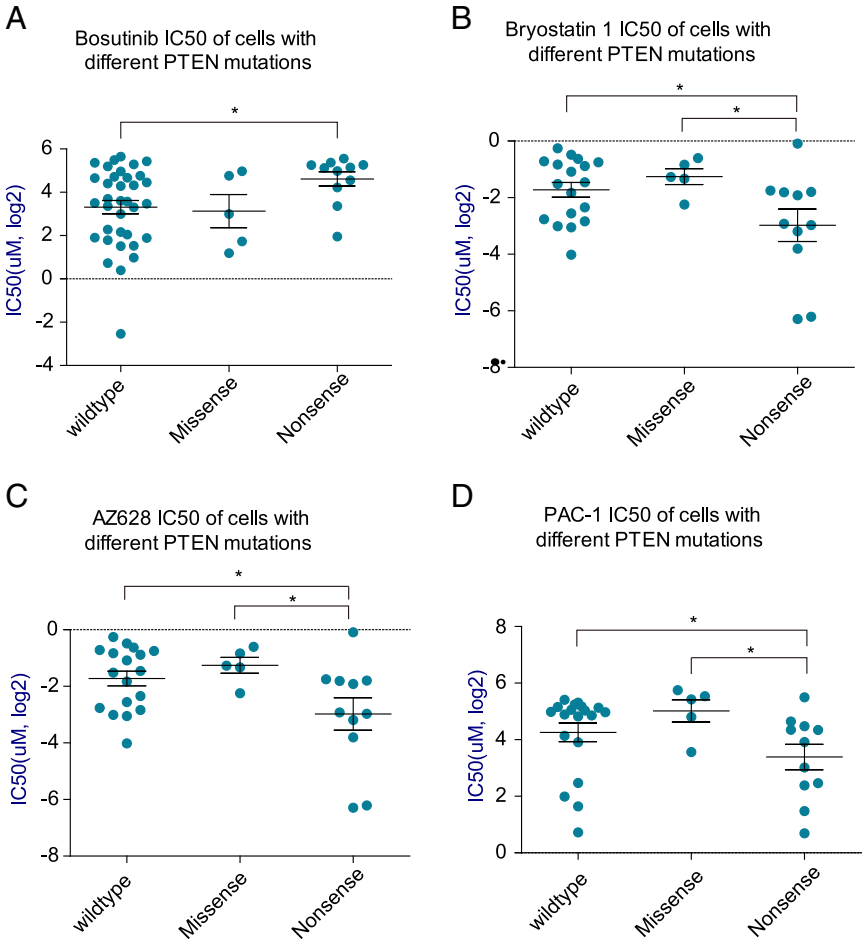


**Figure 4.** Nonsense PTEN mutations confer characteristic drug responses. Comparison between the drug sensitivity of human brain tumor cell lines with different PTEN genotypes. On the basis of CCLE data set, the drug responses (IC50 values) were compared between GBM cases carrying nonsense or missense PTEN mutations using Mann-Whitney test. The nonsense mutations showed increased resistance to bosutinib (A), whereas they displayed favorable responses to bryostratin 1 (B), AZ628 (C), and PAC-1 (D). Star signs indicate significant difference ($P < .05$).

tyrosine- protein/abelson murine leukemia viral oncogene homolog 1 (Src/Abl) tyrosine kinase inhibitor] than missense mutations (Figure 4*A* and Table 1). Because Src inhibitors can reverse Src-induced suppression of PTEN function [24], the ineffectiveness of bosutinib on these cells actually suggested a stronger LOF effect of nonsense mutations over missense mutations.

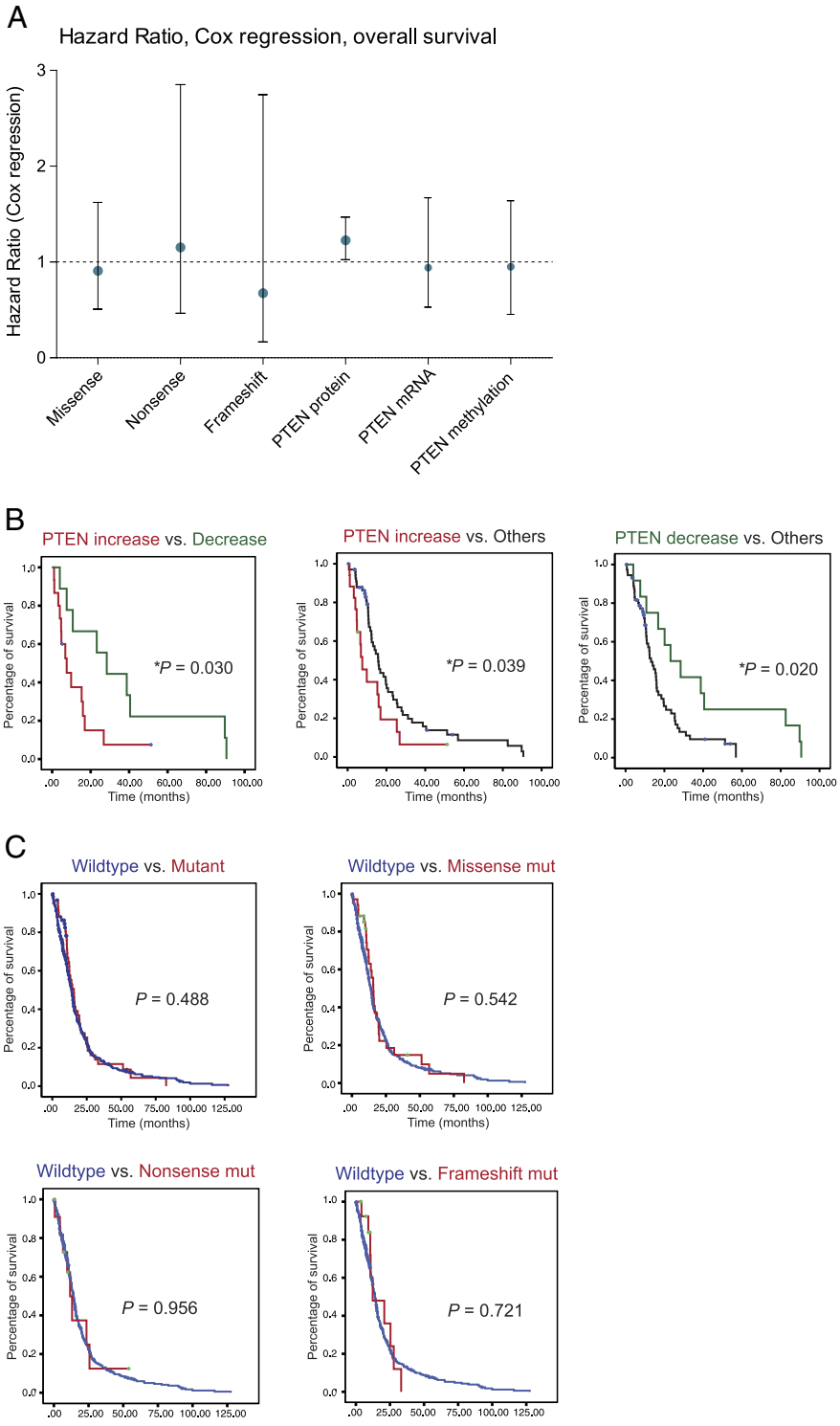


**Figure 5.** Overexpression of Pten protein associates with shorter OS of patients with GBM. (A) Distinct effects of PTEN mutation, expression, and promoter methylation on OS of patients with GBM. Cox regression was used to analyze the effects of abovementioned factors on OS, and the respective HRs are shown (with 95% CIs). Only the increased Pten protein level was found to associate with shorter OS ($P < .05$, star signs). (B) Kaplan-Meier survival test revealed shorter OS for patients with GBM expressing increased level of Pten protein compared to other patients. (C) No association was found between OS and the mutations of PTEN, as shown by Kaplan-Meier analysis. In addition, no difference was found between the OS of patients carrying PTEN missense, nonsense, or frameshift mutations.

Importantly, nonsense mutations of PTEN displayed favorable responses to bryostatin 1 (Sigma), AZ628 (Sigma), and procaspase activating compound-1 (PAC-1, Sigma; Figure 4, *B–D*), suggesting that the adverse effects of nonsense mutations might be targetable. Because PTEN loss causes the activation of protein kinase C (PKC), it is not surprising that bryostatin (PKC inhibitor) can suppress the growth of cells carrying nonsense PTEN mutations. Another adverse consequence of PTEN loss is the cooperation with Ras/Raf/mitogen- activated protein kinases (MAPK) for promoting tumorigenesis [25], and this may explain the enhanced effect of AZ628 (a Raf inhibitor) against nonsense mutations. Finally, loss of PTEN inhibits caspase 3 activity, and this may be the underlying mechanism for the effectiveness of PAC-1 (a caspase 3 activator) on PTEN nonsense mutations. Taken together, the drug sensitivity profile of PTEN nonsense mutations is in good consistency with its severe LOF phenotype and may provide important information for its targeted therapy.

### *Pten Protein Up-Regulation Associates with Shorter Overall Survival*

Furthermore, we tested the effect of PTEN mutation and expression on overall survival (OS) of patients with GBM. Cox regression

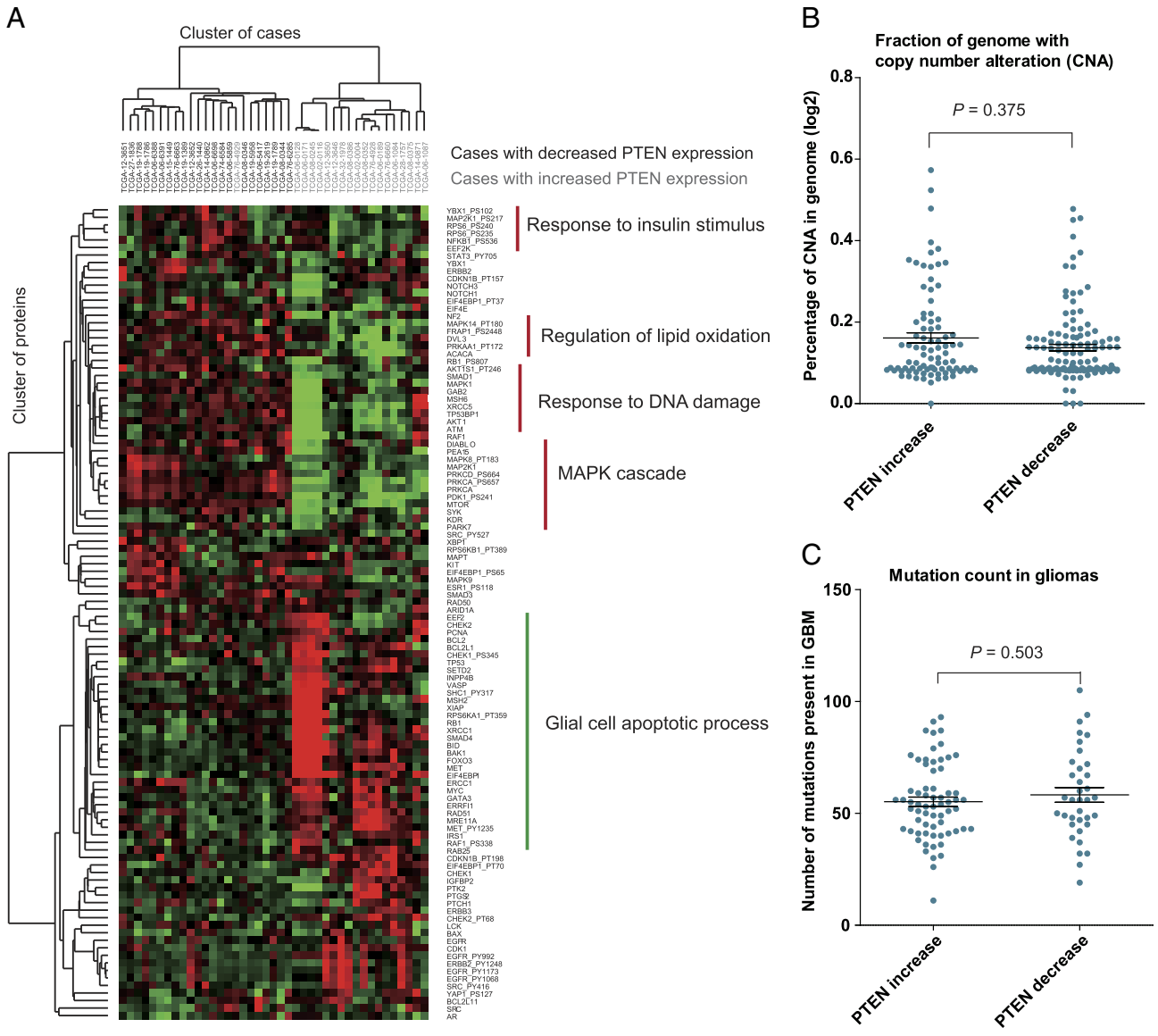


**Figure 6.** Increased Pten protein expression indicates substantial alteration in signaling pathways. (A) Hierarchical clustering of patients with GBM according to the expression of different signaling proteins. The levels of signaling proteins/phosphoproteins belonging to various pathways were obtained from RPPA data set of TCGA, and a hierarchical algorithm (Pearson correlation, average linkage) was used for the clustering. This classification well distinguished the patients with increased Pten protein expression (shorter OS) or with decreased Pten levels, with only one exception of 41 cases. Several clusters of proteins were also identified, and Gene Ontology annotation (Lawrence Berkeley National Laboratory, Berkeley, CA) suggested the activation of pathways involved in response to insulin stimulus, regulation of lipid oxidation, response to DNA damage and MAPK cascade, as well as the decreased activity of glial cell apoptotic pathway. (B and C) Pten protein level is not associated with CNA fraction or number of mutations in the genomes of GBM cases. The CNA fraction (B) and number of mutations (C) were compared between GBM cases expressing increased or decreased levels of Pten protein using Mann-Whitney test, and no significant difference was found between the two groups.

survival analyses revealed a link between increased Pten protein level and shorter OS (HR = 1.23, 95% CI = 1.03-1.47; Figure 5*A*). Patients with upper quarter Pten protein expression displayed significantly shorter OS (median, 7.5 months) than the rest of patients (median, 15.7 months; Figure 5*B*). However, no correlation was found between OS and PTEN mutation, mRNA level or promoter methylation (Figure 5, *A* and *C*).

Interestingly, patients with GBM with unregulated Pten protein showed substantial alterations in signaling pathways involved in insulin stimulus, lipid oxidation, DNA damage and MAPK cascade, and inactivation of cell apoptotic process (Figure 6*A*). The expression level of Pten showed no correlation with CNA fraction in genome or the total number of mutations present in the tumor (Figure 6, *B* and *C*). These findings suggest distinct mechanisms whereby PTEN mutations and altered protein expression affect DFS and OS of patients with GBM.

## Discussion

Although the prognostic value of PTEN in GBM has been controversial, here, we have demonstrated strong association between PTEN mutation/expression and survival of patients with GBM.

The analysis is based on a large number of patients with comprehensive clinical and genomic data, and the combined analysis on genomic stability, signaling pathways, and drug sensitivity provides mechanistic insight into the distinct effects of PTEN mutations. We experimentally validated the effects of PTEN mutations on genomic instability and p53/Gata3 protein levels, thereby confirming the findings in patients with GBM.

Our analysis revealed stable association between upregulated Pten protein and worse outcome of GBM. Patients with upper-quarter Pten protein level showed significantly shorter median survival and higher HR compared to the others, and this association was evident for both OS and DFS ($P < .05$, Cox regression). To our knowledge, this study presents the first analysis on the prognostic value of quantified Pten protein level for survival of patients with GBM. Meanwhile, it should be noted that PTEN mRNA level and promoter methylation were not associated with survival of patients with GBM, and this may explain why previous studies focusing on mRNA or methylation did not report any prognostic significance [26]. Interestingly, GBM with increased Pten protein level displayed substantial alterations in signal- ing pathways involved in DNA damage, MAPK cascade, and cell apoptotic process, which may provide mechanistic explanations for the chemoresistant phenotype and worse prognosis of these patients.

The distinct effects of nonsense and missense mutations of the PTEN gene also add to the complexity of mutational effects of this pivotal tumor suppressor. Nonsense mutations, but not missense or frameshift mutations, were associated with shorter DFS of patients with GBM (median survival time decreased by approximately 50%). Consistently, only nonsense mutations were correlated to the significant increase of mutations in the genome and the potent decrease in p53 and Gata3 protein levels. These findings suggest stronger LOF effects for nonsense mutations and lead to the question whether mutations of PTEN should be equally considered when evaluating their biologic consequences or prognostic significances. In fact, distinct mutational effects have been well characterized for another important tumor suppressor, p53. Hot-spot mutations of p53 confer distinct effects on tumor spectrum and survival of mutant knock-in mouse models [27–29], and these are considered as consequences of different LOF and GOF effects [30,31]. To determine if PTEN mutations also display different strengths of LOF or even GOF effects, both in vitro and in vivo studies should be carried out on the basis of each frequently occurring mutation.

Finally, we show that the survival-shortening PTEN nonsense mutations can be targeted by drugs that inhibit PKC (bryostatin) and Raf (AZ628) or activate procaspase 3 (PAC_1). These findings suggest a link between PTEN genotype and drug sensitivity profile and encourage future studies employing PTEN status as a marker for GBM subclassification and personalized therapeutics.

## References


[1] Ahmadloo N, Kani AA, Mohammadianpanah M, Nasrolahi H, Omidvari S, Mosalaei A, and Ansari M (2013). Treatment outcome and prognostic factors of adult glioblastoma multiforme. *J Egypt Natl Canc Inst* **25**, 21–30.

[2] Song MS, Salmena L, and Pandolfi PP (2012). The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol* **13**, 283–296.

[3] Ming M and He YY (2012). PTEN in DNA damage repair. *Cancer Lett* **319**, 125–129.

[4] Mayo LD and Donner DB (2002). The PTEN, Mdm2, p53 tumor suppressor–oncoprotein network. *Trends Biochem Sci* **27**, 462–467.

[5] Freeman DJ, Li AG, Wei G, Li HH, Kertesz N, Lesche R, Whale AD, Martinez-Diaz H, Rozengurt N, and Cardiff RD, et al (2003). PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and-independent mechanisms. *Cancer Cell* **3**, 117–130.

[6] Rodríguez-Escudero I, Oliver MD, Andrés-Pons A, Molina M, Cid VJ, and Pulido R (2011). A comprehensive functional analysis of PTEN mutations: implications in tumor- and autism-related syndromes. *Hum Mol Genet* **20**, 4132–4142.

[7] Carico C, Nuño M, Mukherjee D, Elramsisy A, Dantis J, Hu J, Rudnick J, Yu JS, Black KL, and Bannykh SI, et al (2012). Loss of PTEN is not associated with poor survival in newly diagnosed glioblastoma patients of the temozolomide era. *PloS One* **7**, e33684.

[8] Kraus JA, Glesmann N, Beck M, Krex D, Klockgether T, Schackert G, and Schlegel U (2000). Molecular analysis of the PTEN, TP53 and CDKN2A tumor suppressor genes in long-term survivors of glioblastoma multiforme. *J Neurooncol* **48**, 89–94.

[9] Ruano Y, Ribalta T, de Lope AR, Campos-Martin Y, Fiaño C, Pérez-Magán E, Hernández-Moneo JL, Mollejo M, and Mélendez B (2009). Worse outcome in primary glioblastoma multiforme with concurrent epidermal growth factor receptor and p53 alteration. *Am J Clin Pathol* **131**, 257–263.

[10] Yang L, Clarke MJ, Carlson BL, Mladek AC, Schroeder MA, Decker P, Wu W, Kitange GJ, Grogan PT, and Goble JM, et al (2008). PTEN loss does not predict for response to RAD001 (Everolimus) in a glioblastoma orthotopic xenograft test panel. *Clin Cancer Res* **14**, 3993–4001.

[11] Minaguchi T, Yoshikawa H, Oda K, Ishino T, Yasugi T, Onda T, Nakagawa S, Matsumoto K, Kawana K, and Taketani Y (2001). PTEN mutation located only outside exons 5, 6, and 7 is an independent predictor of favorable survival in endometrial carcinomas. *Clin Cancer Res* **7**, 2636–2642.

[12] Ermoian RP, Furniss CS, Lamborn KR, Basila D, Berger MS, Gottschalk AR, Nicholas MK, Stokoe D, and Haas-Kogan DA (2002). Dysregulation of PTEN and protein kinase B is associated with glioma histology and patient survival. *Clin Cancer Res* **8**, 1100–1106.

[13] Bäcklund LM, Nilsson BR, Goike HM, Schmidt EE, Liu L, Ichimura K, and Collins VP (2003). Short postoperative survival for glioblastoma patients with a dysfunctional Rb1 pathway in combination with no wild-type PTEN. *Clin Cancer Res* **9**, 4151–4158.

[14] Endersby R and Baker SJ (2008). PTEN signaling in brain: neuropathology and tumorigenesis. *Oncogene* **27**, 5416–5430.

[15] Olive PL and Banáth JP (2006). The comet assay: a method to measure DNA damage in individual cells. *Nat Protoc* **1**, 23–29.

[16] Cancer Genome Atlas Research Network (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* **455**, 1061–1068.

[17] Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, and Larsson E, et al (2013). Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* **6**, p11.

[18] Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehár J, Kryukov GV, and Sonkin D, et al (2012). The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. *Nature* **483**, 603–607.

[19] Braun R, Finney R, Yan C, Chen QR, Hu Y, Edmonson M, Meerzaman D, and Buetow K (2013). Discovery analysis of TCGA data reveals association between germline genotype and survival in ovarian cancer patients. *PLoS One* **8**, e55037.

[20] Eisen MB, Spellman PT, Brown PO, and Botstein D (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* **95**, 14863–14868.

[21] Meyn RE (2009). Linking PTEN with genomic instability and DNA repair. *Cell Cycle* **8**, 2322–2323.

[22] Wan X, Yokoyama Y, Shinohara A, Takahashi Y, and Tamaya T (2002). PTEN augments staurosporine-induced apoptosis in PTEN-null Ishikawa cells by downregulating PI3K/Akt signaling pathway. *Cell Death Differ* **9**, 414–420.

[23] Yu T, MacPhail SH, Banáth JP, Klokov D, and Olive PL (2006). Endogenous expression of phosphorylated histone H2AX in tumors in relation to DNA double-strand breaks and genomic instability. *DNA Repair* **5**, 935–946.

[24] Lu Y, Yu Q, Liu JH, Zhang J, Wang H, Koul D, McMurray JS, Fang X, Yung WK, and Siminovitch KA, et al (2003). Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. *J Biol Chem* **278**, 40057–40066.

[25] Mulholland DJ, Kobayashi N, Ruscetti M, Zhi A, Tran LM, Huang J, Gleave M, and Wu H (2012). Pten loss and RAS/MAPK activation cooperate to promote EMT and metastasis initiated from prostate cancer stem/progenitor cells. *Cancer Res* **72**, 1878–1889.

[26] Camara-Quintana JQ, Nitta RT, and Li G (2012). Pathology: commonly monitored glioblastoma markers: EFGR, EGFRvIII, PTEN, and MGMT. *Neurosurg Clin N Am* **23**, 237–246.

[27] Lang GA, Iwakuma T, Suh YA, Liu G, Rao VA, Parant JM, Valentin-Vega YA, Terzian T, Caldwell LC, and Strong LC, et al (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell* **119**, 861–872.

[28] Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, Crowley D, and Jacks T (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* **119**, 847–860.

[29] Hanel W, Marchenko N, Xu S, Yu SX, Weng W, and Moll U (2013). Two hot spot mutant p53 mouse models display differential gain of function in tumorigenesis. *Cell Death Differ* **20**, 898–909.

[30] Freed-Pastor WA and Prives C (2012). Mutant p53: one name, many proteins. *Genes Dev* **26**, 1268–1286.

[31] Kim E and Deppert W (2007). Interactions of mutant p53 with DNA: guilt by association. *Oncogene* **26**, 2185–2190.

## Supplementary material

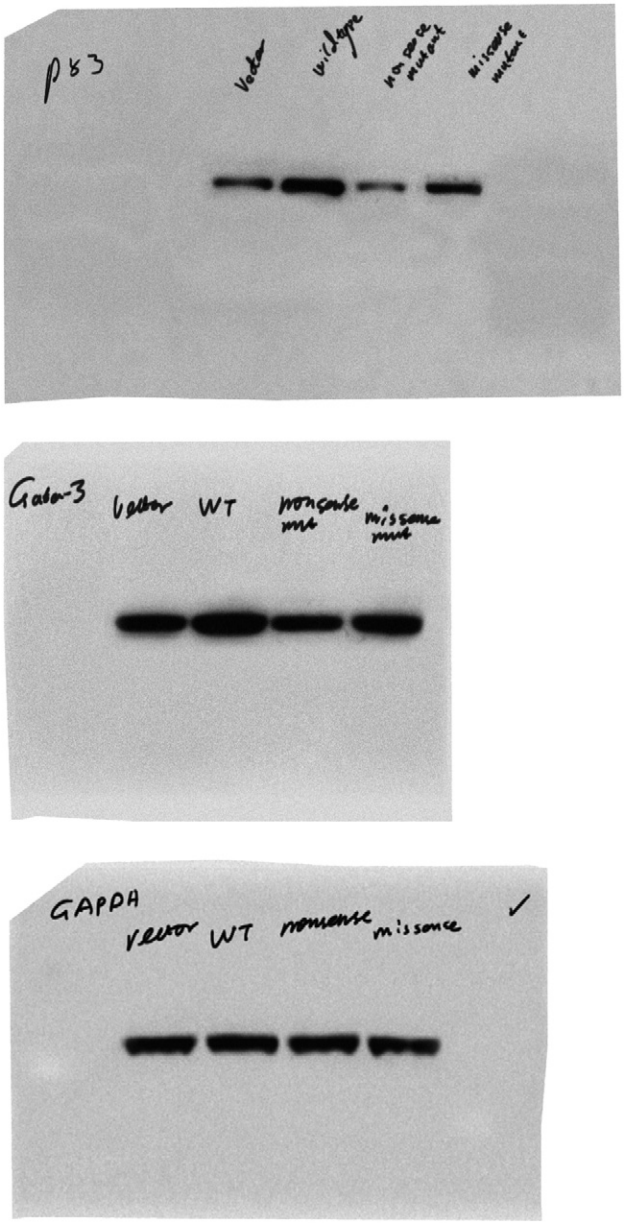


**Figure W1.** The full gels for Figure 2F. The Western Blot shown in Figure 2F for p53, Gata3 and GAPDH are respectively shown below.