Phosphoproteomic analysis with reverse phase protein array identifies N-myc downstream regulated gene 1 (NDRG1) as a biomarker of phosphatidylinositol-3-kinase (PI3K)α inhibitor activity in a *PTEN*-deficient glioma cell line.

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Abstract

Aberrant activation of phosphoinositide 3-kinase (PI3K) signaling is commonly found in cancer, and leads to deregulation of several intracellular processes, including cell survival, growth, proliferation and migration. PI3K activates AKT, serum/glucocorticoid regulated kinase (SGK), phosphoinositide -dependent kinase 1 (PDK1), mammalian target of rapamycin (mTOR), and several other molecules involved in cell progression and survival. Reports have also indicated that the deletion of the gene encoding the tumor suppressor phosphatase and tensin homologue (*PTEN*) is one of the key factors controlling activation of the PI3K pathway. Although many therapies to target the Ras-PI3K-mTOR axis in glioblastoma (GBM) have been attempted, their efficacies have not been pronounced, presumably because of the complexity of PI3K mediated signaling.

To elucidate the role of *PTEN* in PI3K signaling, the phosphorylation status of two glioma cell lines, LN229 (wild type *PTEN*) and U-87 MG (*PTEN* deficient) was investigated with phosphoproteomic reverse phase protein array (RPPA). When basal phosphorylation was compared between both cell lines, the phosphorylation of N-myc downstream regulated gene 1 (NDRG1) (Thr346), a physiological substrate of SGK1, was increased in U-87 MG cells compared with the levels in LN229 cells. Following treatment with PIK-90, a PI3K inhibitor, phosphorylation of Akt (Ser473) and its direct substrate PRAS40 (Thr246) were decreased in both cell lines, however, NDRG1 (Thr346) phosphorylation was strongly inhibited only in U-87 MG cells. These data suggest that *PTEN* deletion affects the phosphorylation status of NDRG1 in U-87 MG cells and the differential effects of PIK-90 on LN229 and U-87 MG cells is *PTEN* dependent. Therefore, detecting NDRG1 (Thr346) phosphorylation could be a biomarker for PI3Kα inhibitor treatment in *PTEN* deleted cancer cells. Ongoing studies are focusing on elucidating the mechanism of action of PIK-90 in glioma cell lines.

Methods

Cell Culture : Two human GBM cell lines, LN229 and U-87 MG cells were incubated in DMEM or MEM respectively, containing 10% FBS. Cells were treated with 0.5μM PIK-90 and harvested immediately and after 2, 6 and 24 hours. PIK-90 is reported to inhibit PI3Kα (IC_{50} = 11nM), PI3Kβ (IC_{50} = 350nM), PI3Kδ (IC_{50} = 58nM) and PI3Kγ (IC_{50} = 18nM)[1].

RPPA : Cells were collected, washed and homogenized in lysis buffer. Serially diluted lysates (1:1, 1:2, 1:4, 1:8) were spotted onto glass slides with an arrayer equipped with 32 pins in order to place the expression level of samples in a dynamic range for signal detection. Each sample dilution series were then spotted in eight replicates. Signals generated from slides stained with anti-phospho antibodies were analyzed employing SuperCurve algorithm to obtain a single value of relative concentration for each lysate to draw the heatmap. For the statistical analysis, eight replicates were separately handled (n=8) and two sample t-test was performed between untreated control and treated samples. The targets of the anti-phospho antibodies utilized in this study are shown in the website (http://www.carnabio.com/english/ images/rppa_antibody_en.pdf?121130) and a paper[2].

[1] http://www.scbt.com/datasheet-364587-CASNumber-677338-12-4.html
[2] Masuda M et al. Mol Cell Proteomics, M113.033845, March 18, 2014.

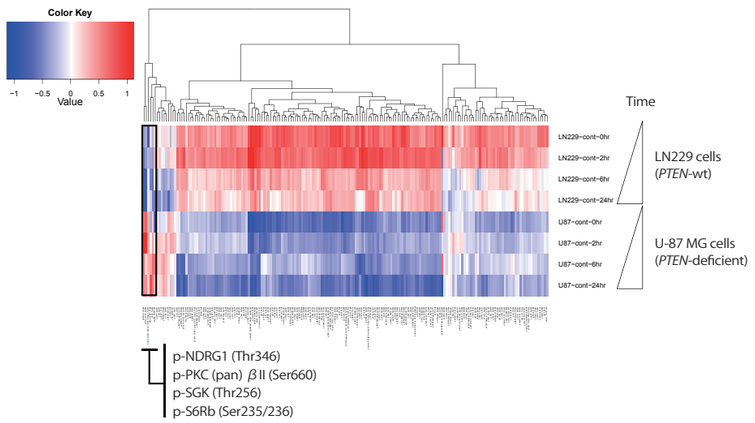

Figure 1. Basal phosphoprotein content in untreated LN229 (*PTEN*-wt) and U-87 MG (*PTEN*-deficient) cells. Most phosphoprotein expressions are higher in LN229 cells except for several molecules in PI3K and mTOR complex 2 (mTORC2) pathway.

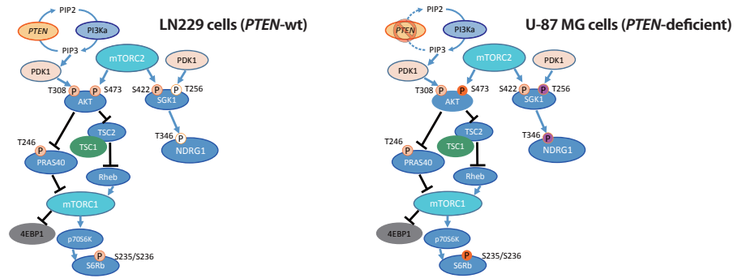

Figure 2. PI3K signaling pathway in LN229 and U-87 MG cells.

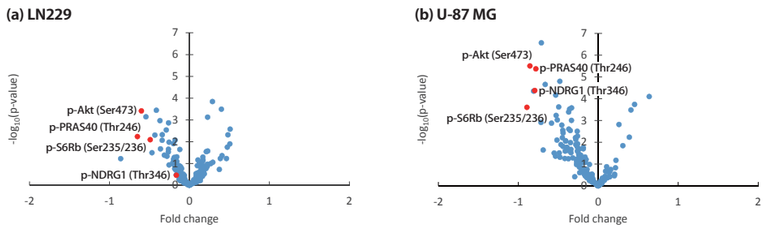

Figure 3. The change in phosphorylation of 180 phosphoproteins in (a) LN229 and (b) U-87 MG cells after 6-hour treatment with PIK90. The phosphoproteins indicated in red exhibited significant change by PIK90 treatment. The change in phosphorylation of NDRG1 (Thr346) was only found in U-87 MG (*PTEN*-deficient) cell line.

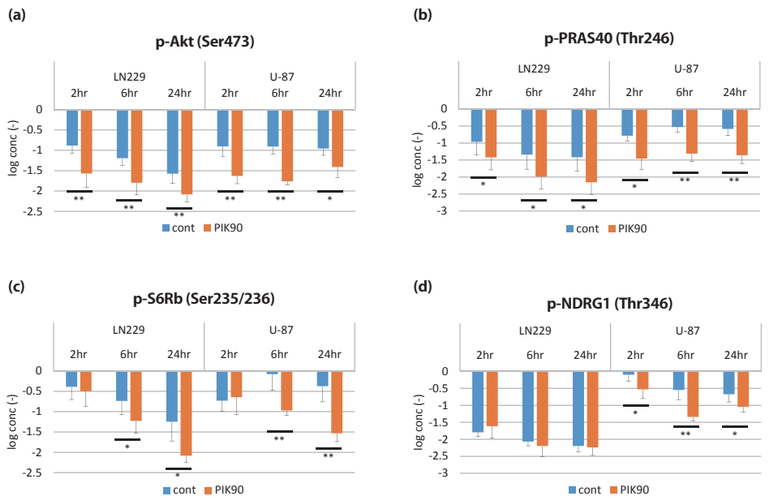

Figure 4. The time course of phosphoprotein expressions in LN229 and U-87 MG cells after exposure to PIK90. The phosphorylation of (a) Akt (Ser473), (b) PRAS40 (Thr246) and (c) S6 ribosomal protein (S6Rb) (Ser235/236) was decreased in both cell lines in response to PIK90 treatment. In contrast, (d) NDRG1 (Thr346) phosphorylation was inhibited only in U-87 MG cells. The inhibition was time dependent-manner up to 24 hours. The values are mean ± s.d. (n=8). cont : untreated control, *: p < 0.05, **: p < 0.001 vs control.

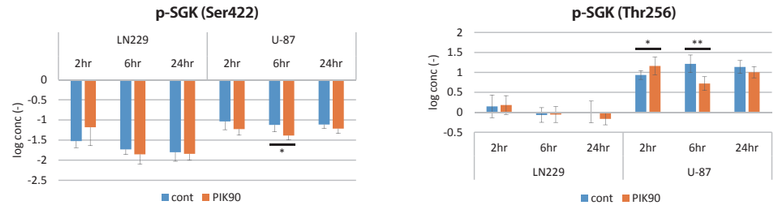

Figure 5. The time course of change in phosphorylation of SGK in LN229 and U-87 MG cells following PIK90 treatment. The phosphorylation of both SGK(Ser422) and SGK (Thr256) was inhibited by treatment with PIK90 in U-87 MG cells. However, the effect was weak comparing with inhibition of NDRG1 (Thr346) phosphorylation. The values are mean ± s.d. (n=8). cont : untreated control, *: p < 0.05, **: p < 0.001 vs control.

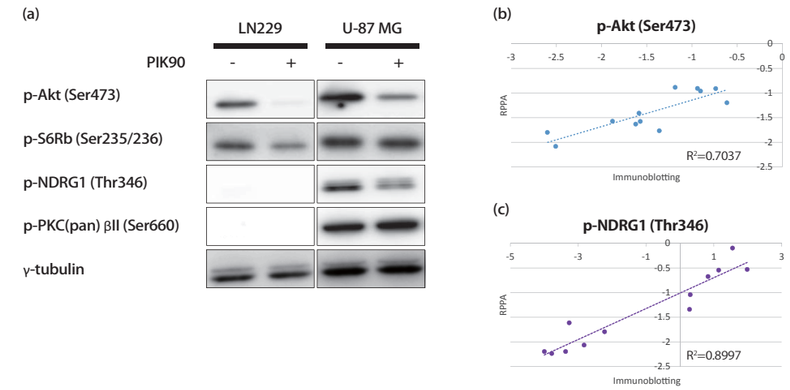

Figure 6. Verification of RPPA results by immunoblotting. (a) Immunoblott analysis of cell lysates prepared after 6-hour incubation with or without PIK90. Relative concentrations as $\log_2$ value from RPPA results showed good correlations with immunoblotting data for (b) p-Akt (Ser473) and (c) p-NDRG1 (Thr346). The latter values are densitometric ratios of the phosphoproteins to the loading control, γ-tubulin.

Conclusion

1. **In LN-299 cells, basal phosphorylation levels were higher than U-87 MG cells. However, PI3K and mTORC2 pathway signals were attenuated.**
2. **In contrast, in U-87 MG cells, PI3K and mTORC2 pathways were activated.**
3. **By treatment with PIK90, among PI3K and mTORC2 pathway, the phosphorylation of NDRG1 (Thr346) was strongly inhibited in U-87 MG cells.**
4. **These results suggest that NDRG1 (Thr346) phosphorylation could be an efficacy biomarker of PI3Kα inhibitor treatment.**
5. **The down-regulation of SGK1 by PIK90 is relatively weak comparing with the suppression of NDRG1 phosphorylation, indicating that NDRG1 phosphorylation would be also controlled by unknown signaling pathways.**
6. **RPPA results showed good correlations with immunoblotting data and RPPA is an appropriate tool for surveying phosphoproteome.**