Evaluation of PSAT1 post-translational modifications in EGFR-mutant lung cancer

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Abstract

Epidermal growth factor receptor (EGFR) is a transmembrane protein that binds the EGF ligand to affect cell growth. It is of particular clinical importance as mutation of the EGFR gene has been shown to accelerate cell growth resulting in lung cancer. EGFR-signaling is promoted through post-translational modifications (PTM), such as phosphorylation or acetylation, of downstream target proteins. These PTMs can affect the activity and/or cellular localization of these targets. Phosphoserine Aminotransferase (PSAT), an enzyme encoded by the PSAT1 gene, is overexpressed in lung cancer and we previously demonstrated that PSAT1 translocates to the nucleus upon EGFR-activation. Thus we hypothesize that EGFR-signaling promotes modifications of PSAT1 that may lead to its nuclear localization. To examine this, we immunoprecipitated Flag-tagged PSAT1 from EGFR-mutant PC9 lung cancer cells treated with or without EGFR-inhibitor, erlotinib, and assessed phosphorylation or acetylation changes by immunoblot. Our results indicate that PSAT1 is neither acetylated or phosphorylated under these conditions. Separately, we explored an additional question on whether PSAT associated with Ezrin, a protein known to influence cytoskeleton actin arrangement. For this, the procedure was modified to include anti-Ezrin antibody upon PSAT pulldown. Controlling for contamination by non-specific association to the IP beads, we conclude that Ezrin is not uniquely associated with PSAT. Future research is necessary to further investigate the relationship between PSAT and EGFR; specifically for other potential PSAT1 modifications or associating proteins.

Methods

Cell culture and treatment
PC9 cells stably transfected to express FLAG-tagged PSAT1 (FLAG-PSAT1-PC9) were cultured in RPMI supplemented with 10% fetal bovine serum, puromycin, hygromycin, and gentamicin. For each experiment, cells were plated in 6 T-75 flasks at 3 million cells per flask. After 24 hours, flasks were treated with either DMSO or 1μM Erlotinib, a recognized inhibitor of EGFR that is clinically used for treatment of EGFR-mutant non-small cell lung cancer, in serum-depleted RPMI. This treatment comparison was performed to step ensure that differences to any observed post-translational modifications could be attributed to the activity of EGFR. Flasks were treated for 24 hours prior to lysate harvest.

Preparation of cell lysate
At the end of treatment, all cells were collected by trypsination and washed twice with ice-cold PBS. Pelleted cells were lysed in 100μL of IP lysis buffer supplemented with protease and phosphatase inhibitors on ice for 10 minutes. Lysates were further disrupted by passaging through insulin syringe followed by centrifugation to remove cellular debris. Protein concentration was performed by BCA assay against a standard curve.

Immunoprecipitation
For IP, 900 ug of protein was incubated with 25 uL of washed FLAG-M2 beads in 1mL of buffer with constant rotation overnight at 4°C. A portion of lysate was also set aside to serve as lysate input for SDS-PAGE. Following incubation, samples were loaded onto magnet and lysates were discarded. Beads were thoroughly washed 3X with 500 uL of 1X Tris solution. FLAG-PSAT1 was eluted from beads by incubation with 125ng/uL of FLAG-peptide in 40 uL of 1X Tris for 30 minutes with constant rotation. Eluted proteins were mixed with 4X SDS loading buffer in preparation for SDS-PAGE.

SDS-PAGE
First, 20 ug of lysate input was mixed with 6 uL of 4X loading buffer. Lysate inputs and IP eluted protein samples were heated at 100°C for 5 minutes followed by quick centrifugation. Protein ladder, lysate, and IP samples were loaded on a 7.5% PAGE gel and proteins were separated by SDS-PAGE at 200 volts for 35 minutes. Proteins were then transferred to PVDF membrane at 200 volts for 1 hour on ice. After transfer, PVDF membrane was blocked in 5% non-fat dry milk in TBS-Tween for one hour on orbital shaker.

Western Blotting
Blocked PVDF membranes were incubated overnight with rocking at 4°C with either mouse anti-acetylated lysine, anti-phospho Ser/Thr antibody, rabbit anti-PSAT1, or rabbit anti-Ezrin at 1:1000 dilution in 5% BSA in TBS-Tween. Membranes were then washed three times for 10 minutes with TBS-Tween followed by incubation with either Trueblot anti-mouse or anti-rabbit HRP secondary antibody (1:5000 to 1:40000 dilution) for 1 hour at room temperature. Protein detection was performed using enhanced chemiluminescence reagent and radiological film exposure in the dark room. For PTM analysis, membranes were first probed using the anti-acetyl or anti-phospho antibodies followed by anti-PSAT1 to confirm immunoprecipitation. For protein association, membranes were first probed with the anti-Ezrin antibody followed by anti-PSAT1 to confirm immunoprecipitation.

Acknowledgments

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Results

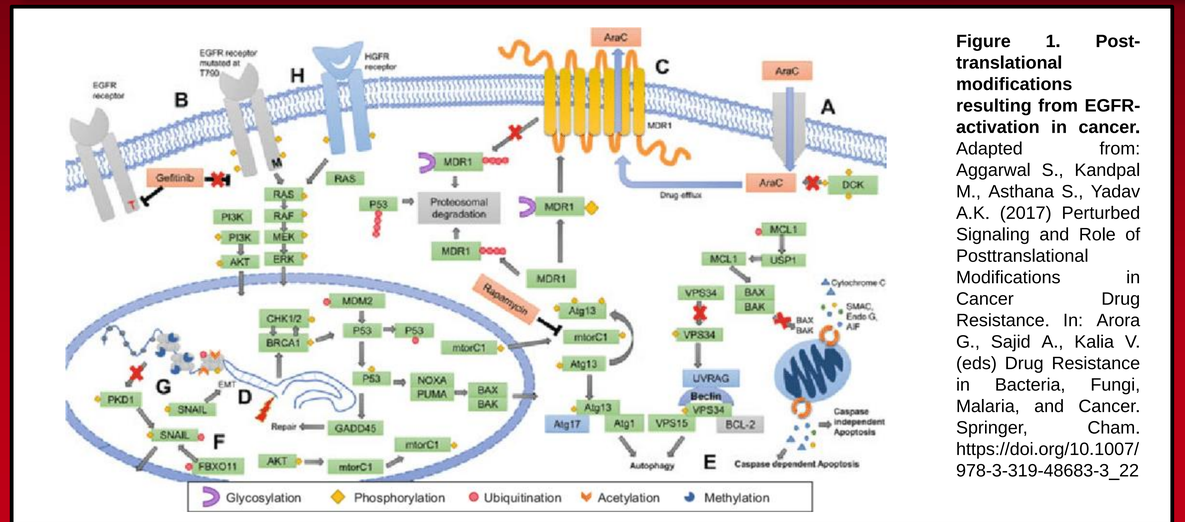


Figure 1. Post-translational modifications resulting from EGFR-activation in cancer. Adapted from: Aggarwal S., Kandpal M., Asthana S., Yadav A.K. (2017) Perturbed Signaling and Role of Posttranslational Modifications in Cancer Drug Resistance. In: Arora G., Sajid A., Kalia V. (eds) Drug Resistance in Bacteria, Fungi, Malaria, and Cancer. Springer, Cham. https://doi.org/10.1007/978-3-319-48683-3_22

Figure 2. EGFR-activation leads to nuclear translocation of PSAT1 in NSCLC cells. EGFR-mutant PC9 cells stably expressing control or PSAT1 shRNA were treated with 1 μM of erlotinib for 48 hrs. Cytoplasmic and nuclear fractions were examined by immunoblot analysis using anti-PKM2 and PSAT1 antibodies. OCT1 and α-tubulin served as loading controls for nucleus, and cytoplasm, respectively. Shown is representative images from three separate experiments.

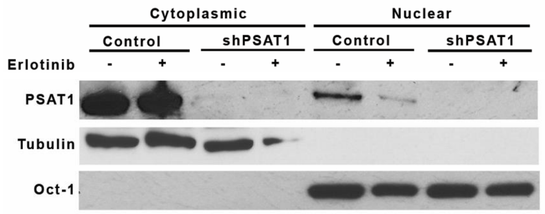
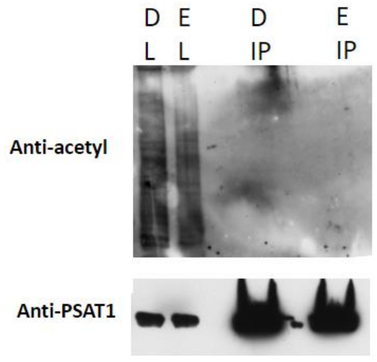


Figure 3: Initial confirmation of PSAT immunoprecipitation from PC9 cell lysates.

Immunoblot analysis of immunoprecipitates from non-treated FLAG-PSAT1-PC9 cells was used to determine the feasibility of pull-down of FLAG-PSAT1 using FLAG-M2 magnetic IP beads. Membrane was probed using anti-PSAT1 antibody.

Figure 4: PSAT1 appears not to be acetylated in PC9 cells.

Lysates (L) or immunoprecipitates (IP) from FLAG-PSAT1-PC9 cells treated with DMSO (D) or Erlotinib (E) were assessed by immunoblot using anti-acetyl-lysine or anti-PSAT1 antibodies. Lysates were used to confirm antibody activity against acetylated lysine containing proteins and for qualitative assessment of efficiency of the PSAT1 pull-down. Shown is representative of two separate experiments.



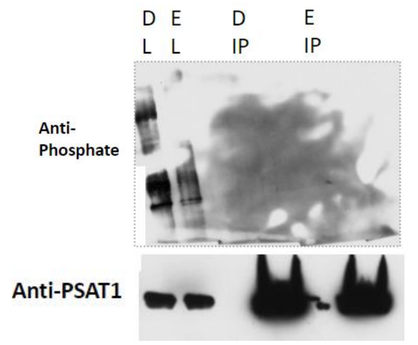
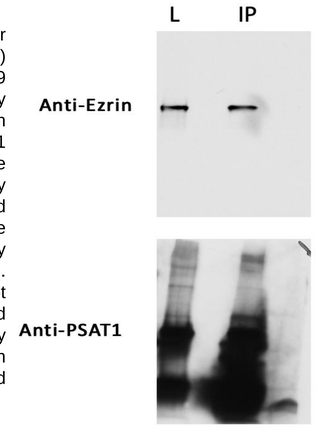


Figure 5: PSAT1 appears not to be phosphorylated in PC9 cells.
Lysates (L) or immunoprecipitates (IP) from FLAG-PSAT1-PC9 cells treated with DMSO (D) or Erlotinib (E) were assessed by immunoblot using anti-phospho Ser/Thr or anti-PSAT1 antibodies. Lysates were used to confirm antibody activity against Ser/Thr phosphorylated containing proteins and for qualitative assessment of efficiency of the PSAT1 pull-down. Shown is representative of two separate experiments.

Figure 6: Initial examination of PSAT1:Ezrin association in PC9 cells.

Lysates (L) or immunoprecipitates (IP) from FLAG-PSAT1-PC9 were assessed by immunoblot using ezrin or anti-PSAT1 antibodies. Lysates were used to confirm antibody activity against ezrin and for qualitative assessment of efficiency of the PSAT1 pull-down. Use of non-Trueblot secondary antibody led to observation of heavy and light immunoglobulin bands seen at 50 and 25kDa, respectively.



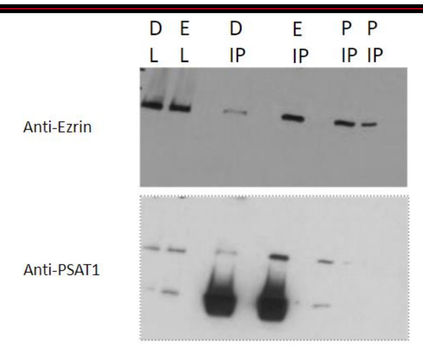


Figure 7: Ezrin binds non-specifically to FLAG M2-beads.
Lysates (L) or immunoprecipitates (IP) from FLAG-PSAT1-PC9 cells treated with DMSO (D) or Erlotinib (E) were assessed by immunoblot using anti-ezrin or anti-PSAT1 antibody. The inclusion of parental (P) PC9 cell IP sample was done to control for non-specific interaction with the M2-beads as as these cells do not express FLAG-PSAT1 (n = 1).

Conclusions

- PSAT1 appears to be neither acetylated or phosphorylated in EGFR-mutant PC9 cells.
- Ezrin is not uniquely associated with PSAT.

Future Directions

- Assess other PTMs such as tyrosine phosphorylation.
- Define other associating proteins that may be responsible for PSAT1 cellular localization and its pro-tumorigenic role to identify potential therapeutic targets against NSCLC.