Poster Note 64603

Quantitative Analysis of AKT/mTOR Pathway using Immunoprecipitation and Targeted Mass Spectrometry

Bhavin Patel, Suzanne Smith, Alex Behling, Kaitlyn Conner, Ryan Bomgarden, Kay Opperman, Barbara Kaboord, John Rogers
Thermo Fisher Scientific, Rockford, IL, USA

Overview

Purpose: Identification and quantitation of multiple AKT/mTOR pathway proteins using an optimized immunoprecipitation to mass spectrometry (IP-MS) workflow in two human carcinoma cell lines.

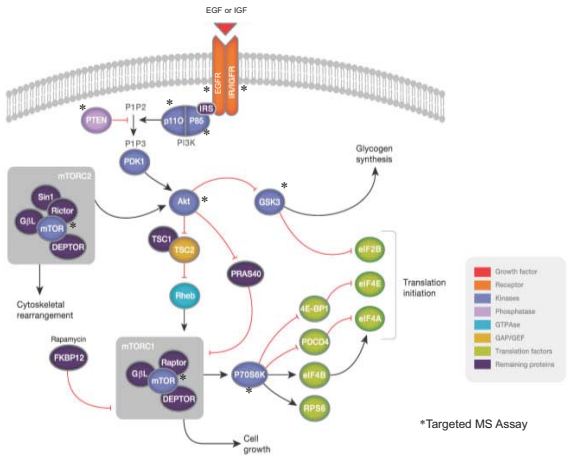
Methods: Serum starved A549 and HCT116 cells were stimulated with EGF or IGF. Several AKT/mTOR pathway targets were enriched by improved IP using Protein A/G and Streptavidin magnetic beads, and IP eluates were processed using in-solution digestion for LC-MS/MS analysis. Targeted MS assays were developed for quantitation of AKT/mTOR pathway target peptides (EGFR, IR, IGF1R, AKT2, AKT1, PTEN, PIK3CA, PIK3R1, mTOR, GSK3α and p70S6K). Multiple targets were then immunoprecipitated simultaneously and quantitated using the targeted MS assays using either PRM or SRM mode. Additionally, improved IP combined with targeted MS workflow was applied to assess recovery of recombinant proteins in a human plasma matrix.

Results: Previously, we assessed over 13 different commonly used resins for IP and determined that magnetic particle supports had the lower background, higher recovery and better reproducibility compared to agarose, sepharose and ultralink supports. In this study, we validated numerous antibodies for IP-MS and optimized specific wash buffers for both Pierce Protein A/G and Streptavidin magnetic beads. Multiplex IP enrichment of total and phos EGFR, AKT, PI3K, PTEN, IR, p70S6K, mTOR and GSK3α resulted in quantitation of low to sub fmol concentrations in two cell lysates by nanoLC-SRM/MS and nanoLC-PRM/MS. In addition, we used IP-MS to enrich and quantify recombinant EGFR, AKT2/AKT1 and PI3K spiked into a human plasma matrix and were able to quantify targets as low as 10ng/ml. As our improved IP-MS workflow results in higher yield of target protein and less non-specific binding, we were able to combine multiple target antibodies to enrich multiple AKT/mTOR pathway protein targets for a single IP-MS analysis. This multiplex targeted assay can be used for simultaneous detection and quantitation of AKT/mTOR pathway proteins in other cancer cell lines or tissues.

Introduction

PI3K/AKT/mTOR pathway plays a central role in tumor progression and anti-cancer drug resistance. The quantitative measurement of protein expression and PTMs of the AKT/mTOR pathway is necessary for precisely characterizing the disease, monitoring cancer progression and determining treatment responses.[1] A major limitation in the quantitation of AKT/mTOR pathway proteins is the lack of rigorously validated methods/reagents and a reliance on semi-quantitative results from Western blotting. MS is increasingly becoming the detection methodology of choice for protein abundance and PTMs. IP is commonly used upstream of MS as an enrichment tool for low-abundant protein targets.[2,3] In addition to protein identification, IP can be combined with targeted MS to identify proteins of interest and protein-protein interactions.[4]

FIGURE 1. PI3K-AKT-mTOR pathway.



Methods

Cell Culture

A549 and HCT116 cells were grown in F-12K (Hamm's) and McCoy's 5A Media, respectively, with 10% FBS/1xPenStrep to ~70% confluency. Cells were starved in 0.1% charcoal stripped FBS for 24 hours before stimulation with 100ng/ml of IGF or EGF for 15 minutes.

Immunoprecipitation and MS Sample Preparation

The Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kit (Protein A/G) was used to screen and validate antibodies for EGFR, total AKT, phospho AKT, PTEN, PIK3CA, IGF1R, IR, mTOR, GSK3α and p70S6K from 500µg cell lysate. Validated antibodies were biotinylated with the Thermo Scientific™ Pierce Antibody Biotinylation Kit for IP. The Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kit (Streptavidin) was used to perform the single or multiplex IPs for target enrichment. IP samples were processed by an in-solution digestion method where IP eluates were reconstituted in 6M Urea, 50mM TEAB, pH 8.5 followed by reduction, alkylation and trypsin digestion overnight at 37°C. The digested samples were acidified with TFA before MS analysis.

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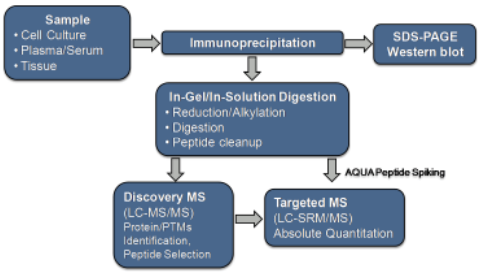
Liquid Chromatography and Mass Spectrometry

Prior to MS analysis, tryptic digest samples were desalted on-line using the Thermo Scientific™ Acclaim™ PepMap 100 C18 Trap Column. For discovery MS, the samples were analyzed by nanoLC-MS/MS using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System and Thermo Scientific™ Orbitrap Fusion™ Mass Spectrometer. For targeted MS, the samples were analyzed using the UltiMate 3000 RSLCnano System and the Thermo Scientific™ TSQ™ Vantage™ Mass Spectrometer (SRM mode) or the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (PRM mode).

MS Data Analysis

Discovery MS data were analyzed with Thermo Scientific™ Proteome Discoverer™ 1.4 and Proteome Software Scaffold 4.0 to assess percent sequence coverage, unique peptides, MS1 intensities, spectral counts and PTMs. The Proteome Discoverer software searches were executed using the Uniprot human protein database. The following search parameters were used: two missed cleavages permitted, carbamidomethylation on cysteine fixed modification, and oxidation (methionine), acetyl (N-terminus proteins) and phosphorylation (S, T, Y) variable modifications. The mass tolerances for precursor ions and fragment ions were 20 ppm and 0.6 Da, respectively. Tryptic peptides with highest MS1 intensity and relevant phosphorylation sites were selected from the discovery data for targeted assay development. For targeted MS data analysis, Thermo Scientific™ Pinpoint software and Skyline software (University of Washington) were used to measure limit of quantitation (LOQ) from the calibration curve and target analyte concentration from unknown samples.

FIGURE 2. Experimental workflow for IP-MS assay development.



Protein targets are immune-enriched from matrix and analyzed by silver stain or Western blot after gel electrophoresis. IP samples are also digested with trypsin and analyzed by nanoLC-MS/MS to identify candidate quantitative peptides. Heavy isotope-labeled, quantitative peptide standards are then used in targeted MS assays for absolute quantitation.

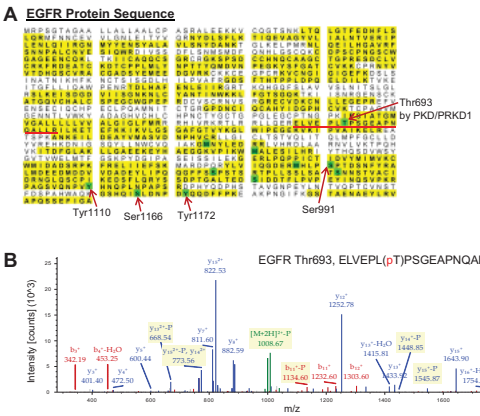
Results

FIGURE 3. Enrichment of medium to low abundant AKT-mTOR pathway targets from A549 cells.

IP Antibody	Targets Identified	Neat No. of Unique Peptides	IP Enriched No. of Unique Peptides		Relevant Phosphopeptide Identified
			Unstimulated	+ IGF or EGF	
EGFR	EGFR	14	41	+IGF: 42	+IGF: Thr693, Ser991, Ser1166
				+EGF: 51	+EGF: Ser991, Tyr1110, Tyr1172
Phospho AKT	AKT1	-	3	20	+IGF: Ser473
	AKT2	-	-	14	+IGF: Ser474
	AKT3	-	-	13	N/A
AKT1	AKT1	-	16	12	N/A
	AKT2	-	9	11	N/A
	AKT3	-	5	3	N/A
PTEN	PTEN	-	5	10	N/A
PIK3CA	PIK3CA	-	-	7	N/A
	PIK3R1	-	-	5	N/A
	PIK3R2	-	-	4	N/A
Phospho mTOR	mTOR	2	75	82	+IGF: Thr2446, Ser2448
	RICTOR	-	0	2	N/A
	SIN1	-	2	3	N/A
	Gbl	-	4	4	N/A

AKT-mTOR pathway targets were immunoprecipitated from unstimulated and IGF or EGF-stimulated A549 lysate with Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kits (Protein A/G or Streptavidin) for MS analysis. Higher number of unique peptides were identified in IP enriched samples compared to neat lysate. Protein isoforms and interacting partners were identified for AKT, PIK3CA and mTOR targets. Relevant phosphorylation sites were detected for EGFR, AKT1, AKT2 and mTOR. Candidate quantitative peptides were selected for targeted MS assay development.

FIGURE 4. Identification of multiple phosphorylation sites for EGFR peptides in IGF or EGF-stimulated A549 cells.



A) IP-MS of IGF or EGF stimulated A549 lysate allowed simultaneous analysis of multiple phosphorylation sites for EGFR. Identified peptides are highlighted in yellow and modified amino acids (S, T, Y) are in green. EGF-stimulation allowed detection of phosphorylation at Ser991, Tyr1110 and Tyr1172. Phosphorylation at Thr693, Ser991 and Ser1166 were identified with IGF stimulation.

B) Representative MS/MS spectra of EGFR Thr693 phosphopeptide ELVEPL(pT)PSGEAPNQALLR.

Immunoprecipitation to Targeted MS Application (nLC-SRM/MS or nLC-PRM/MS)

FIGURE 5. Detection and quantitation limits of EGFR, AKT2, AKT1, PTEN, PIK3CA and PIK3R1 peptides.

Target	Peptide No.	LOD (fmol)	LLOQ (fmol)	ULOQ (fmol)	Linearity (R^2)
EGFR	Total Peptide	0.2	3.9	1000	0.9977
	Phospho Peptide	0.2	3.9	1000	0.9997
AKT2	Peptide 1	0.2	3.9	1000	0.9998
	Peptide 2	3.9	15.6	1000	0.9599
AKT1	Peptide 1	0.2	3.9	1000	0.9541
PTEN	Peptide 1	0.2	3.9	1000	0.9999
	Peptide 2	0.2	3.9	1000	0.9997
PIK3R1	Peptide 1	0.2	3.9	1000	0.9997
	Peptide 2	0.2	3.9	1000	0.9999
PIK3CA	Peptide 1	0.2	3.9	1000	0.9981

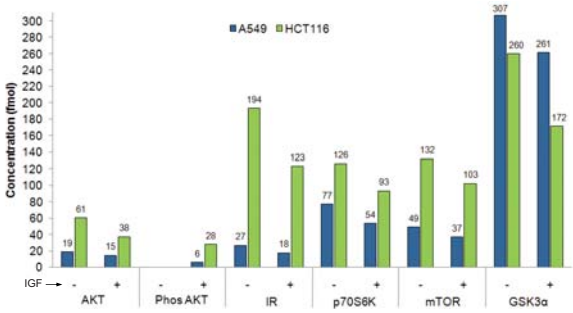
All six targets were monitored with linear quantitation ranging from 3.9 to 1000 fmol.

FIGURE 6. Multiplex immunoprecipitation to MS applications.

IP Antibody	Quantotypic Peptide	nanoLC-MS/MS No. of Unique Peptides		nanoLC-SRM/MS Concentration (fmol)	
		Control	+ EGF	Control	+ EGF
EGFR	Total EGFR	41	51	323.8	446.4
	Phos EGFR			7.8	18.4
AKT1	AKT1	9	8	31.6	42.4
	AKT2	–	4	37	47.8
PTEN	PTEN	2	6	33.2	47.2

EGFR, AKT isoforms and PTEN were enriched simultaneously from unstimulated and EGF stimulated A549 lysates with biotinylated antibodies and Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kit (Streptavidin). All five targets were identified by nanoLC-MS/MS and quantified in low to sub-fmol concentrations by nanoLC-SRM/MS. Phospho-EGFR peptide showed more than two fold increase in response to EGF stimulation.

FIGURE 7. Multiplex IP of IR-AKT-mTOR pathway proteins and nanoLC-PRM/MS.



Multiplex IP enrichment of AKT (Total & Phos), IR, p70S6K, mTOR, and GSK3α from unstimulated and IGF stimulated A549 and HCT116 lysates with Thermo Scientific™ Pierce MS-Compatible Magnetic IP Kit (Streptavidin). Total targets were quantified in low to sub-fmol concentrations by nanoLC-PRM/MS. Up-regulation of phos AKT was seen after IGF stimulation. MS data correlated with subsequent ELISA and Luminex assay results. (Data not shown.)

FIGURE 8. Recovery of recombinant rEGFR, rAKT1, rAKT2 and rPIK3R1 proteins from plasma matrix.

IP Antibody	Matrix	No. of Unique Peptides	Quantified by nanoLC-SRM/MS
EGFR	10ng rEGFR/500µg Plasma	13	9fmol/ 1.2ng
Pan AKT	10ng rAKT1/500µg Plasma	9	9fmol/ 0.5ng
	10ng rAKT2/ 500µg Plasma	4	9fmol/ 0.5ng
PIK3R1	10ng rPIK3R1/500µg Plasma	6	3fmol/ 0.25ng

rEGFR, rAKT1, rAKT2 and rPIK3R1 spiked into 0.5mg plasma were detected and quantitated in low fmol/nanogram concentrations.

FIGURE 9. Summary of PI3K/AKT/mTOR pathway targets identified and quantified in two cell lines without and with enrichment.

Target	Cell line	Detected by Orbitrap Fusion		Quantified by SRM/PRM	
		Neat	Enriched-IP	Neat	Enriched-IP
EGFR	A549	+ (14)*	+ (41)	–	+
	HCT116	+ (10)	+ (34)	–	+
AKT1	A549	–	+	–	+
	HCT116	–	+	–	+
AKT2	A549	–	+	–	+
	HCT116	–	+	–	+
PIK3CA	A549	–	+	–	+
	HCT116	–	+	–	+
PIK3R1	A549	–	+	–	+
	HCT116	–	+	–	+
PTEN	A549	–	+	–	+
	HCT116	–	+	–	+
mTOR	A549	+ (2)	+ (82)	–	+
	HCT116	+ (9)	+ (110)	–	+
IGF1R	A549	+ (4)	+ (22)	–	+
	HCT116	–	+	–	+
IR	A549	–	+	–	+
	HCT116	–	+	–	+
GSK3α	A549	+ (5)	+ (23)	–	+
	HCT116	+ (6)	+ (23)	–	+
p70S6K	A549	–	+	–	+
	HCT116	–	+	–	+

+ ≥2 unique peptides for discovery MS and >LLOQ for targeted MS
* No. of unique peptides

Conclusion

- Immunoprecipitation using MS-Compatible Magnetic IP Kits (Protein A/G and Streptavidin) for MS applications resulted in a higher yield of AKT/mTOR pathway target proteins and less non-specific binding proteins.
- IP to MS analysis of EGFR, total AKT, phospho AKT, PIK3CA and phospho mTOR enabled identification of multiple isoforms, relevant protein interactions and phosphorylation sites.
- Multiplex IP to targeted MS allowed simultaneous quantitation of PI3K-AKT-mTOR pathway proteins in the low to sub fmol range from unstimulated and stimulated A549 and HCT116 cell lysates.
- Enrichment of as low as 10ng recombinant EGFR, AKT2, AKT1 and PIK3R1 proteins spiked in plasma enabled absolute quantitation by targeted MS.
- Enrichment is necessary for identification and quantitation of low abundant signaling pathway proteins and PTMs for MS applications.
- Future work will focus on development of targeted MS assay for quantitation of additional AKT/mTOR pathway targets.

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