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Identification of Phosphoproteins Regulated by Protein Phosphatase 2A Inhibition in Human Hepatocellular Carcinoma

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Serine/Threonine Protein Phosphatase 2A (PP2A) serves as an exquisite switch in controlling several key oncogenic and kinase signalling pathways involved in cell proliferation, apoptosis, migration and invasion (1). While much is known about kinase regulation of oncogenic pathways, little is known about the role of PP2A. In this study we use a phosphoproteomics approach to investigate the role PP2A inhibition plays in altering protein class function in hepatocellular carcinoma (HCC).

Human hepatocellular carcinoma cells (Hep3B) were treated with 40nM okadaic acid in serum free conditions for 24h. Phosphoproteins were isolated using a Phosphoprotein Enrichment & Purification Kit (Clontech, USA), cleaned on a 10% SDS-PAGE gel and stained with coomassie blue (0.25%). Protein bands were excised, digested in-gel with trypsin and analysed by nLC-ESI-MS/MS. The MS/MS data files generated were analysed using Mascot 2.1 software. Three or more matching peptides and a significant probability score ($P<0.001$) were required for a secure identity assignment. All MASCOT search results were inspected manually to ensure correct matches by bioinformatics, while 5 phoshoproteins were confirmed by western blot. PhosphoSitePlus™ was used to predict serine/threonine phosphorylation sites in the proteins. Classification of identified proteins was completed using PANTHER Gene Ontology™ to categorise all identified proteins based on their biological function. Proteins were identified using gene nomenclature. Ingenuity Pathway Analysis (IPA) was used to investigate molecular networks affected by PP2A inhibition. Hep3B were tested for their ability to aggregate using hanging drop suspension cultures following exposure to okadaic acid and photographed on an EVOS® FL Cell Imaging System.

352 phosphoproteins were identified in Hep3B cells by mass spectrometry under basal conditions. Following exposure to okadaic acid, 180 new phosphoproteins and the loss of 5 basal expressed proteins were identified. These new phosphoproteins were associated with nucleic acid binding (24%), cytoskeleton (10.4%), transcription factors (6.8%), signalling (2.1%), cellular junction (2.1%), cell adhesion (2.6%), and enzyme modulation (8.9%). Pro-metastatic proteins such as PAK2, AKT2, PLCB3, CTNNA1, MLC, RUVL1, HSPA14 and ROCK2 were all detected following phosphatase inhibition. Major phosphorylation motifs identified included MAPKAPK1 motif, AMP-activated protein kinase motif, CLK1 motif, GSK-3 motif and ERK1 and ERK2 motifs. ROCK2, MSN, eif3H, HYOU1 and VASP expression were confirmed by western blot. IPA software revealed MYC as the major upstream regulator, while RhoA and EIF2 signaling were identified as top canonical pathways. Following exposure to okadaic acid, Hep3B spheroids showed a decrease in cell-cell interaction and increase in cellular motility in comparison to their retrospective controls.

Our results indicate that Ser/Thr phosphatase inhibition leads to the activation of protein networks involved in tumour growth, metabolism, cellular communication and invasion. Activation of both Rho and EIF2 signaling in response to PP2A inhibition is key in the proliferation and metastasis of HCC. Many new anti-cancer drugs target kinase and phosphatase controlled pathways; our data highlight the intricacy and diversity of cellular functions protein phosphatases control and further elucidate their complexity as potential therapeutic targets.

(1) Shi Y (2009). Serine/threonine phosphatases: mechanism through structure. *Cell* 139(3): 468-484.