Mapping c-Src Phosphorylation Sites as Potential Disease Biomarkers

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Protein post-translational modifications (PTMs) are essential in regulation of many physiological processes and dysregulation of these modifications is usually indicative of disease states. Therefore, mapping PTMs in signaling proteins has become a powerful tool to discover biomarkers for disease diagnosis and progression. The non-receptor tyrosine kinase, c-Src, has been implicated in the development of numerous human diseases including cancers. c-Src is a key nodal signal transducer, playing critical roles in phosphorylating target proteins and regulating different signal transduction pathways. In recent years, accumulating evidence has demonstrated that c-Src plays an important role in regulating G protein coupled receptor (GPCR) signaling, but little is known about how GPCR activation regulates c-Src activity via phosphorylation on c-Src. Here, we characterized the effects of β2 adrenergic receptor (β2AR) stimulation on the regulation of c-Src phosphorylation using on-line LC/MS/MS.

A HEK-293 cellline stably over-expressing hemagglutinin (HA)-tagged c-Src was used for this study. The β2ARs were stimulated with full agonist isoproterenol (ISO). After Iso stimulation, the HA-c-Src proteins were isolated from cell lysate, and subjected to trypsin digestion. The resultant tryptic peptides were subjected to Fe(3)-IMAC (immobilized metal affinity chromatography) for phosphopeptide enrichment. The enriched phosphopeptides were analyzed on a Thermo Scientific LTQ Orbitrap XL mass spectrometer system online coupled with a Waters nanoAcquity UPLC. The LTQ Orbitrap XL was operated in the data dependent mode using the TOP10 strategy. Following ISO stimulation, many phosphorylation sites throughout the c-Src protein sequence were identified (i.e., S12, S17, S35, S37, S39, S51, S69, S70, T72, T74, T75, T87, T88, Y93, S104, S183, S187, S212, S216, S218, S219, S225, S232, Y338, Y419, Y420, Y439). While some of these identified phosphorylation sites correspond to known sites identified under other experimental conditions, most have not been reported previously and were categorized as β2AR (ISO)-specific phosphorylation sites. Based on the MS/MS data, several tryptic peptides were phosphorylated on different sites, indicating the presence of differential phosphorylation states of c-Src in response to Iso stimulation of β2AR.

These identified phosphorylation sites will be used to develop targeted MS assays for quantifying c-Src phosphorylation. Targeted MS by multiple reaction monitoring (MRM) will be developed and first validated using the HEK-293 cellline stably over-expressing HA-tagged c-Src. Once validated, these MRM-based assays will be used to quantify the phosphorylation states of c-Src in different human cancer biopsies. Normal human biopsy tissues will be used as controls. Biomarkers will be discovered by compared the data between cancer and control samples.

This study should add substantially to our knowledge of c-Src phosphorylation and the relationships between these modifications and GPCR activation, and should help to develop new biomarkers for cancer diagnosis and progression. In addition, MRM methods are readily transferrable to other tissues and across instrument platforms. Finally, these studies should demonstrate the scalability of this approach for novel biomarker discovery.