Enrichment and Six-Plex Profiling of the DNA Damage Response Pathway Using Amine- and Cysteine-Reactive Tandem Mass Tag Reagents

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Overview

Purpose: To identify and characterize the DNA damage-response proteins in pulmonary carcinoma A549 cells in response to five different anti-cancer agents.

Methods: Six-plex, amine-reactive Tandem Mass Tag (TMT) reagents were used for total proteome labeling and relative quantitation among treatments. Six-plex cysteine-reactive Tandem Mass Tag (cysTMT) reagents were used for subproteome peptide labeling followed by enrichment using a novel immobilized anti-TMT antibody resin.

Results: Using both amine- and cysteine-reactive TMT reagents, we identified over 4700 peptides (FDR < 1%) and quantitated the relative abundance of over 1000 proteins of A549 cells treated with DNA-damaging agents. However, only changes in the most abundant DNA damage-regulated proteins were observed in un-enriched samples labeled with TMT. CysTMT reagent-enriched samples resulted in the identification and quantitation of additional proteins whose levels were altered after drug treatment.

Introduction

The DNA damage-response pathway is critical in maintaining genome stability, and proteins within this pathway are commonly mutated or mis-regulated in cancer cells (Figure 1).[1] We used a mass spectrometry (MS)-based proteomic approach to identify and characterize the DNA damage-response proteins in pulmonary carcinoma A549 cells in response to five anti-cancer agents: camptothecin, etoposide, actinomycin D, hydroxyurea, and paclitaxel (Figure 2). Each of these drugs has been used clinically to treat cancer and has distinct mechanisms of action. Camptothecin and etopiside are topoisomerase II and I inhibitors, respectively, and prevent unwinding of DNA during replication in S-phase. Actinomycin D, an RNA polymerase II inhibitor, and hydroxyurea, a dNTP synthesis inhibitor, are also S-phase inhibitors. Paclitaxel is a microtubule stabilizer that arrests cells in mitosis. We used TMT reagents to determine relative changes in overall protein abundance after each drug treatment.

Tandem Mass Tag reagents enable concurrent identification and multiplexed quantitation of proteins in different samples using tandem MS.[2] Isobaric TMT reagents share identical structures, including a peptide-reactive labeling group, a spacer arm and an MS/MS reporter. During MS/MS analysis, each isobaric tag produces a unique reporter ion that enables quantitation. Current amine-reactive NHS-ester duplex or six-plex isobaric TMT reagents covalently attach to the free amino termini and lysine residues of peptides and proteins. Our cysTMT reagents and an anti-TMT antibody resin provide an approach of thiol labeling, affinity enrichment, and quantitation similar to isotope-coded affinity tags (ICAT™).[3] Overall, targeted cysteine labeling and affinity enrichment using cysTMT reagents results in reduced sample complexity and improved proteome depth compared to the amine-labeled proteome.

Methods

Sample Preparation

Cell treatment and Western blot: A549 cells grown in DMEM with 10% FBS were treated with 10 µM camptothecin, 200 µM etoposide, 2 µM actinomycin D, 10 mM hydroxyurea, or 10 µg/ml paclitaxel for 20 hours at 37 ºC. A549 cell lysates (10 µg) were separated by SDS-PAGE and blotted using Thermo Scientific p53 antibodies diluted 1:10,000.

TMT® reagent labeling: Control or treated A549 cells were sonicated in lysis buffer (6 M urea/ 50 mM Tris/ 5 mM EDTA) and reduced with 5 mM DTT for 1 hour at 50 ºC and alkylated with 375 mM iodoacetamide for 15 minutes at room temperature. Each sample was acetone precipitated and enzymatically digested at 37 ºC overnight. Peptides were labeled with the appropriate TMTzero® or TMTsixplex® reagent (126-131) for 2 hours at room temperature, quenched with hydroxylamine, and combined before liquid chromatography (LC)-MS/MS analysis.

cysTMT® reagent labeling and enrichment: Treated A549 cells were lysed and reduced as above. Reductant was removed using Thermo Scientific Zeba 7K Desalting Columns, and 100 µg of each sample was immediately combined with 20 µL of 25 mM cysTMT reagent. Each sample was labeled with the appropriate cysTMTzero® or cysTMTsixplex® reagent (126-131) for 2 hours at room temperature, combined, and separated by 6%-12% SDS-PAGE. Eight gel bands were separately enzymatically digested at 37 ºC overnight before extraction and lyophylization. Labeled peptides were resuspended in TBS and incubated with 50 µL of immobilized anti-TMT antibody resin per gel band overnight with end-over-end shaking at 4 ºC. After collecting the unbound sample, the resin was washed four times with 4 M urea/TBS, four times with 0.05% CHAPS, four times with TBS, and four times with water. Peptides were eluted four times with 50% acetonitrile, 0.4% TFA and vacuum-dried before LC-MS/MS analysis.

LC/MS

A NanoLC-2D™ high-pressure liquid chromatograph (HPLC) (Eksigent) with a Proteopep™II C18 column, 75 µm ID x 20 cm (New Objective) was used to separate peptides using a 4%-40% gradient (A: water, 0.1% formic acid; B: acetonitrile, 0.1% formic acid) at a flow rate of 250 nL/min for 60 min. A Thermo Scientific LTQ Orbitrap XL ETD mass spectrometer was used to detect peptides using a top 2 x 3 experiment consisting of single-stage MS, followed by acquisition of three MS/MS spectra with higher-energy C-trap dissociation (HCD) fragmentation, followed by three MS/MS with collision induced dissociation (CID) for protein identification. Parameters in this mode were: Isolation width: 3.0 *m/z*; Collision energy: 50% (10% with two steps). Only doubly- and triply-charged peptides were selected for fragmentation. Dynamic exclusion parameters were set to: Repeat count = 1; Repeat duration = 30; Exclusion list size = 500; Exclusion duration = 20. Target values were as follows: MS = 5 e5; MS/MS (HCD) = 1 e5. Ion transfer times were set to 500 for FTMS and 300 for MS/MS (HCD). Two microscans were required for HCD spectra.

Data analysis: Mixed CID and HCD data were processed with Thermo Scientific Proteome Discoverer™ 1.2 software against the IPI human database using a 20 ppm mass tolerance. Static modifications were set to the TMTsixplex (229.16 Da) or cysTMTsixplex reagent (304.18 Da) and dynamic modifications included phosphorylation and methionine oxidation.

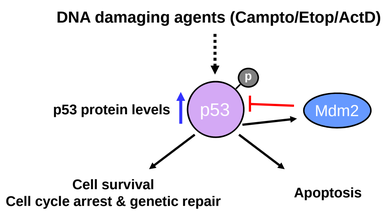
Results

Multiple genomic and proteomic studies have identified roles for p53 in regulation of cell cycle arrest, DNA repair, and apoptosis. Activation of the p53 DNA damage-response pathway causes p53 stabilization through phosphorylation, leading to transcription of various p53-regulated proteins (Figure 1). Treatment of A549 cells with five anti-cancer agents, camptothecin, etoposide, actinomycin D, hydroxyurea, and paclitaxel resulted in varying degrees of p53 activation (Figure 2B), which is consistent with their potency and mechanism of action.

To determine relative changes in abundance of other proteins affected by drug treatment, we used both amine- and cysteine-reactive TMT reagents (Figure 3). Six-plex amine-reactive TMT reagents were used for total proteome labeling of peptides derived from each treatment. A set of six-plex cysTMT reagents were used to label the cysteine-containing subproteome. In contrast to amine-reactive TMT reagents, these tags were used to label denatured proteins before sample mixing and enzymatic digestion. Because of the relatively low abundance of cysteine-containing peptides, approximately 6% of peptides are labeled, allowing for potential sampling of lower abundant proteins. Enrichment of cysTMT reagent-labeled peptides was facilitated by an immobilized anti-TMT antibody resin, which has affinity for the TMT reagent reporter region (Figure 3).

Using these parallel workflows, we were able to identify over 4700 peptides and quantify over 1000 proteins (Figure 2C and 4). Only 541 proteins were similar between the amine- and cysteine-labeled samples, demonstrating increased sample diversity after enrichment. Unique to the cysTMT enriched sample, were some known and novel DNA damage-response proteins, which resulted in changes in relative abundance after drug treatment. Specifically, two DNA damage signaling targets, ASC-1 and aslin 1, were reduced over two-fold in response to camptothecin, etoposide and actinomycin D (Figure 5A, 5B). A similar reduction occurred for the DNA helicase MCM complex, with subunits 2-6 being quantified after enrichment (Figure 5C and data not shown). Some proteins such as the HUWE1, a p53 E3 ubiquitin ligase like MDM2, had increased expression upon drug treatment (Figure 5D). Others such as the small GTPase guanidine exchange factor RalBP1, had drug-specific down regulation (Figure 5E).

FIGURE 1. Model of p53 DNA damage response pathway.



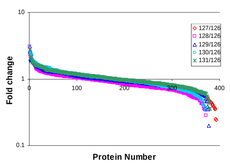
Activation of the p53 DNA damage-response pathway results in p53 phosphorylation and stabilization through interference with MDM2-mediated degradation. Increased p53 levels result in increased expression of stress response genes that promote cell survival through cell cycle arrest or DNA damage repair. p53 activation also induces expression of pro-apoptotic genes and the MDM2 ubiquitin E3 ligase which in turn modulates the down-regulation of the pathway.

FIGURE 2. A549 cell drug treatments and response.

A

Drug	[ ]	Cell cycle phase	TMT reporter
No treatment	-	-	126
Camptothecin	10 µM	S	127
Etoposide	200 µM	S	128
Actinomycin D	2 µM	S	129
Hydroxyurea	10 mM	S	130
Paclitaxel	10 µg/ml	M	131

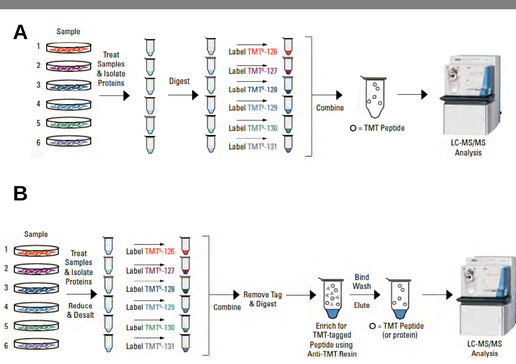
C



B

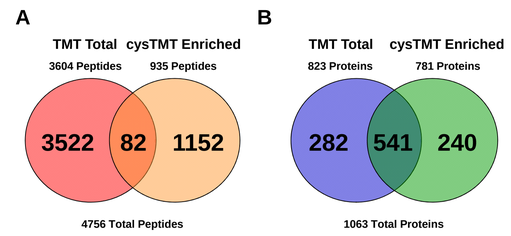
(A) Table of different anti-cancer agents and concentrations used for A549 cells treatment, the cell cycle phase of drug action, and the TMTsixplex or cysTMTsixplex reagents used to label each sample. (B) Western blot of p53 after drug treatment. (C) Log graph of relative quantitation of 400 cysTMT reagent-labeled proteins normalized to no treatment control.

FIGURE 3. Schematic of TMT and cysTMT reagent workflows.



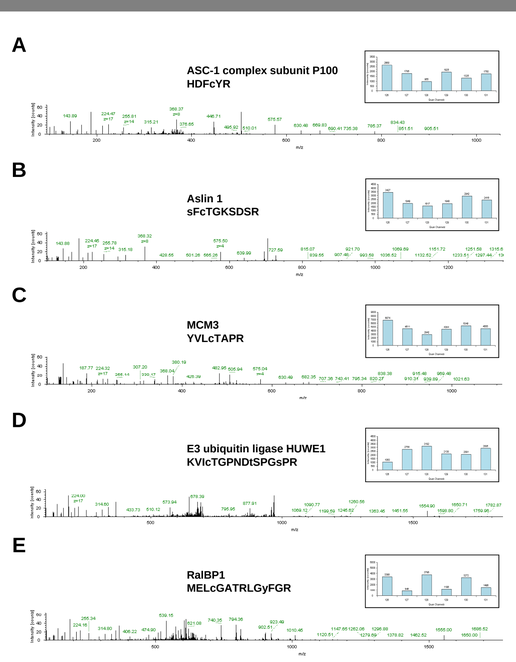
(A) Six-plex TMT reagent workflow for peptide labeling after sample treatment and digestion. (B) Six-plex cysTMT reagent workflow for protein labeling followed by digestion and labeled peptide enrichment using immobilized anti-TMT antibody resin.

FIGURE 4. Comparison of TMT and cysTMT reagent-enriched peptides and proteins.



Venn diagrams depicting the number of unique labeled peptides (A) and proteins (B) identified using Scaffold 3 Q+ (Proteome Software) for TMT reagent-labeled samples and cysTMT reagent sample post enrichment of A549 cell extract using immobilized anti-TMT resin.

FIGURE 5. Identification and quantitation of Thermo Scientific cysTMT reagent-labeled peptides.



(A-E) MS spectra of enriched cysTMT-labeled cysteine-containing peptides and TMT reporter quantitation showing changes in relative abundance of peptides after each drug treatment.

Conclusions

- Amine- and cysteine-reactive Tandem Mass Tags are complementary reagents that facilitate more in-depth proteomic analysis.
- cysTMT reagents combined with immobilized anti-TMT resin enrichment allow for relative quantitation of lower abundant proteins.
- Six-plex TMT reagents enabled simultaneous relative quantitation of five different anti-cancer drugs.
- Combined CID and HCD spectra processing facilitated enhanced quantitative peptide identification.

References

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