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An investigation of heat shock protein 27 and P-glycoprotein mediated multi-drug resistance in breast cancer using liquid chromatography-tandem mass spectrometry-based targeted proteomics

*Feifei Xu, Ting Yang, Danjun Fang, Qingqing Xu, Yun Chen**

School of Pharmacy, Nanjing Medical University, 818 Tian Yuan East Road, Nanjing 211166, China

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ABSTRACT

One missing puzzle piece to study heat shock protein 27 (HSP27) in P-glycoprotein (P-gp) mediated multi-drug resistance (MDR) was the amount of HSP27 and the extent of its phosphorylation in the biological context. Liquid chromatography–tandem mass spectrometry (LC/MS/MS)-based targeted proteomics allows researchers to monitor associated proteins and their modification simultaneously and quantitatively. In this study, a targeted proteomics assay was first developed and validated for the quantification of HSP27 and its phosphorylated forms. Using this assay, the level of HSP27 was determined in non-tumoral cells MCF-10A, parental drug-sensitive cancer cells MCF-7/WT and drug-resistant cancer cells MCF-7/ADR. A decrease of HSP27 expression was observed in P-gp overexpressed MCF-7/ADR cells. A quantitative time-course analysis of both HSP27 and P-gp in doxorubicin (DOX)-treated MCF-7/WT cells also implied that HSP27 may participate in the P-gp modulation. Furthermore, stoichiometry of site-specific HSP27 phosphorylation indicated that DOX treatment rapidly induced the HSP27 phosphorylation at Ser82. Moreover, conventional analytical methods were also performed for a comparison.

Biological significance

LC/MS/MS-based targeted proteomics turns out to be a promising quantification approach for the study of proteins in the preclinical and clinical environment. Unfortunately, rare studies applied this technology to detect multiple associated proteins or protein modification in one experiment. This study demonstrated the potential of LC/MS/MS-based targeted proteomics to understand the cell events in a more accurate context of biological system. By the quantitative time-course analysis of HSP27 and its phosphorylated forms at sites of Ser15 and Ser82, the possible role of HSP27 in P-gp mediated MDR was suggested. Further development of targeted proteomics in future may provide more insight into signal transduction pathways upon perturbation of a protein network or changes to a panel of proposed biomarkers in a given disease state.

* *Corresponding author*. Tel.: +86 25 86868326; fax: +86 25 86868467.
E-mail address: ychen@njmu.edu.cn (Y. Chen).

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1. Introduction

Multi-drug resistance (MDR) is a major obstacle to a successful treatment of breast cancer. To date, several mechanisms have been suggested to explain MDR acquisition [1]. Among them, drug efflux due to increased expression and activity of ATP-binding cassette (ABC) transporters including P-glycoprotein (P-gp), is the most frequently proposed one [2]. However, it is not well known how drugs induce functional ABC transporters in cancer cells. In a variety of suggested factors, heat shock protein 27 (HSP27) has been implicated in this process [3].

HSP27 belongs to a family of small stress proteins with various cellular functions [4]. Since the functional activities of protein are also regulated by its phosphorylation [5], three serine phosphorylation sites of HSP27 (Ser15, Ser78 and Ser82) have been widely concerned [6]. While aberrant level [7,8] and induced phosphorylation [9] of HSP27 have been suggested in MDR acquisition, the exact role of this protein in MDR has not been clearly understood thus far.

As most researchers seek to address the signal transduction pathways between HSP27 and ABC transporters, important information that they can hardly provide is the amount of proteins in the biological system. Currently, the level of proteins has been widely measured using antibody-based methods. While these techniques provide valuable information on protein levels, they often lack the necessary specificity and reproducibility [10]. In addition, the obtained results are potentially complicated by the fact that the protein may exist in several forms (e.g., phosphorylated and non-phosphorylated forms) [11]. The availability and quality of phospho-specific antibodies could be another limitation [12]. More importantly, most of the assays that have been developed are qualitative or semi-quantitative, but not quantitative [13]. As Method of the Year 2012, liquid chromatography–tandem mass spectrometry (LC/MS/MS) based targeted proteomics allows researchers to quantify proteins with high sensitivity, high selectivity and wide dynamic ranges [14]. The underlying principle of this targeted analysis is the specific detection and determination of a protein of interest at the peptide level [15,16]. Peptides are generated by proteolytic digestion of the target protein to serve as surrogate analytes. Selected or multiple reaction monitoring (SRM or MRM) is used to detect the selected surrogate peptide, exploiting the capabilities of triple quadrupole mass spectrometers [17].

To date, a few LC/MS/MS-based targeted proteomics assays have been developed for the quantification of proteins [18,19]. However, rare studies applied this technology to detect multiple associated proteins in one experiment, for example, to look at what happens to protein levels upon perturbation of a protein network, while it is one of the most inherent abilities of targeted proteomics. Thus, we took advantage of this feature to investigate the role of HSP27 in P-gp mediated MDR in the biological context. In the presence of the established assay for P-gp [20], a LC/MS/MS-based targeted proteomics assay for the quantification of HSP27 and its phosphorylated forms at Ser15 and Ser82 was first developed and validated in this study. This assay was then applied to the quantitative analysis of HSP27 in non-tumoral cells MCF-10A, parental drug-sensitive cancer cells MCF-7/WT and drug-resistant cancer cells MCF-7/ADR. As a well-known chemotherapy drug, doxorubicin (DOX) was selected for P-gp induction in MCF-7/WT cells. With the treatment of DOX, the association of HSP27 and its phosphorylation stoichiometry with P-gp was monitored in a time manner. The outcome was compared with those obtained by conventional analytical methods including confocal microscopy, Western blotting and flow cytometry.

2. Materials and methods

2.1. Chemicals and reagents

Phospho(peptides) and internal standard containing stable-isotope labeled amino acids were developed by ChinaPeptides Co., Ltd. (Shanghai, China). Purity of the peptides was also provided by the manufacturer. The stable isotope-labeled amino acid was supplied by Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). HSP27 was purchased from Novus Biologicals (Littleton, CO, USA). Ammonium bicarbonate (NH_4HCO_3) was obtained from Qiangshun Chemical Reagent Co., Ltd. (Shanghai, China). DL-dithiothreitol (DTT), iodoacetamide (IAA) and Tris–HCl were supplied by Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) was obtained from Sinopharm Chemical Reagent Company (Shanghai, China). Sequencing grade modified trypsin was purchased from Promega (Madison, WI, USA). Phosphate buffered saline (PBS) was purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). Acetonitrile (ACN) and methanol were obtained from Tedia Company, Inc. (Fairfield, OH, USA). Trifluoroacetic acid (TFA) and formic acid (FA) were provided by Aladdin Chemistry Co., Ltd. (Shanghai, China) and Xilong Chemical Industrial Factory Co., Ltd. (Shantou, China), respectively. Dulbecco's Modified Eagle Media (DMEM) and fetal bovine serum were obtained from Thermo Scientific HyClone (Logan, UT, USA). Dulbecco's modified Eagle's medium/Nutrient mixture F12 Ham's liquid media (DMEM/F12) was from Thermo Scientific HyClone (Logan, UT, USA). Penicillin was supplied by CSPC Zhongnuo Pharmaceutical Co., Ltd (Shijiazhuang, China). Streptomycin was obtained from Merro Pharmaceutical Co., Ltd (Dalian, China). Trypan blue and sodium dodecyl sulfate (SDS) were obtained from Generay Biotech Co., Ltd (Shanghai, China). Water was purified and deionized with a Milli-Q system manufactured by Millipore (Bedford, MA, USA).

2.2. Preparation of stock solutions, calibration standards and quality controls (QCs)

Since the assay for P-gp has been previously described [20], only the method for HSP27 will be provided in detail. Stock solutions (1 mg/mL) were prepared by accurately weighing the peptides and dissolving them in deionized water. The solutions were stored at −20 °C in a brown glass tube to protect it from light. The corresponding isotope-labeled synthetic peptides were used as internal standards. The

internal standards were also weighed, and 5 μg/mL stock solutions were prepared in deionized water. Internal standard solution (1000 ng/mL for non-phosphorylated peptides and 100 ng/mL for phosphorylated peptides) was prepared by diluting the stock solutions with an ACN:water mixture (50:50, v/v) containing 0.1% FA.

The calibration standards were prepared by serial dilution of the stock solution using HSP27 depleted cell extract as the matrix. Experimental details for preparation of the immuno-depleted matrix are given in the Supplementary material. The concentrations of the calibration standards were 100, 200, 500, 1000, 2500, 5000 and 10,000 ng/mL for each of non-phosphorylated peptides in a mixture. Similarly, phosphopeptides were combined in proportions to create standards that contain 10, 20, 50, 100, 250, 500 and 1000 ng/mL of each phosphorylated peptide. The QC standards for lower limit of quantification (LLOQ), low QC, mid QC and high QC of non-phosphorylated peptide were prepared at 100, 300, 1000 and 8000 ng/mL and those of phosphorylated peptides were at 10, 30, 100 and 800 ng/mL in the same matrix and frozen prior to use.

2.3. Cell culture, immunoprecipitation of HSP27 and protein extraction

The MCF-7/WT (ATTC, Manassas, VA) and MCF-7/ADR (Keygen Biotech, Nanjing, China) cells were cultured in a DMEM media supplemented with 10% fetal bovine serum, 80 U/mL penicillin and 80 μg/mL streptomycin at 37 °C and 5% CO_2. MCF-10A cells (ATTC, Manassas, VA) were non-tumorigenic breast epithelial cells and maintained routinely in DMEM/F12 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were split every 5–7 days by lifting cells with 0.25% trypsin, and feeding between splits was accomplished through the addition of fresh medium. To maintain a highly drug-resistant cell population, MCF-7/ADR cells were periodically reselected by growing them in the presence of 1000 ng/mL DOX [21]. Experiments were performed using the cells incubated without DOX for 48 h and cells were counted with a hemocytometer (Qiujing, Shanghai, China). Cell viability was assessed by trypan blue (0.4%) exclusion, which was completed by mixing the cell suspension, trypan blue and 1 × PBS in a 2:5:3 ratio and counting the percentage of viable cells following a 5 min incubation at 37 °C.

Cells were pelleted at 1480 ×*g* for 10 min. The sample was then split. Half was used for membrane extraction of P-gp [20], and the other half was used for general protein extraction of HSP27. For HSP27 extraction, the cells were pelleted at 1480 ×*g* for 10 min and resuspended in 400 μL of RIPA lysis buffer (Beyotime Institute of Biotechnology, China) containing a protease inhibitor cocktail (Sigma-Aldrich, MO, USA) and a phosphatase inhibitor cocktail (Sigma-Aldrich, MO, USA). After an incubation period of 45 min on ice, the samples were spinned at 16,000 ×*g* for 10 min to remove insoluble material. Then, rabbit polyclonal anti-HSP27 antibody (Epitomics, Burlingame, CA, USA) bound to BioMagPlus Goat anti-Rabbit IgG beads (Bangs Laboratories, Fisher, Indiana, USA) was added to the cell lysate. After the removal of nonspecifically bound proteins, HSP27 was eluted from the beads by heating at 95 °C for 5 min and with a 1% SDS solution. Protein concentrations of the obtained elution were determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

2.4. In-solution tryptic digestion

A quantity of 100 μL of each sample was mixed with 50 μL of 50 mM NH_4HCO_3. Subsequently, the protein was reduced by an addition of 50 mM DTT until a final concentration of 10 mM was achieved. The sample was then incubated at 60 °C for 20 min. The sample was then alkylated by adding 400 mM IAA to obtain a final concentration of 50 mM and incubated at room temperature for 6 h in the dark. Finally, sequencing grade trypsin was added, and the sample was incubated at 37 °C for 24 h. The reaction was stopped by adding 10 μL of 0.1% TFA. Then, 100 μL of the internal standard solution was added to the tryptic peptide mixture before transferring it into an Oasis HLB cartridge (60 mg/3 mL; Waters, Milford, MA, USA) that was preconditioned with 3 mL ACN and 3 mL deionized water. After the sample was loaded, the cartridge was washed with 2 mL of water and 2 mL of ACN:water (50:50, v/v) and eluted with 1 mL of 100% ACN. Finally, the eluent was evaporated to dryness, and the sample was resuspended in 100 μL of ACN:water (50:50, v/v) containing 0.1% FA.

2.5. LC/MS/MS method development and validation

An Agilent Series 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) and a 6410 Triple Quad LC/MS mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) were used for the LC/MS/MS studies.

The liquid chromatography separations were performed on a hypersil gold column (3 μm, 20 mm × 2.1 mm; Thermo Fisher Scientific, USA) at room temperature. The mobile phase consisted of solvent A (0.1% FA in water) and solvent B (0.1% FA in methanol). A linear gradient with a flow rate of 0.3 mL/min was applied in the following manner: B 10% (0 min) → 10% (1 min) → 90% (4 min) → 90% (8 min) → 10% (9 min). The injection volume was 10 μL.

The mass spectrometer was interfaced with an electrospray ion source and operated in the positive MRM mode. Q1 and Q3 were both set at unit resolution. The flow of the drying gas was 10 L/min and the drying gas temperature was held at 350 °C. The electrospray capillary voltage was optimized to 4000 V. The nebulizer pressure was set to 35 psi. The data were collected and processed using the Agilent MassHunter Workstation Software (version B.01.04).

Method validation involves evaluating the linear range, accuracy, precision, limit of quantification (LOQ) and stability. The detailed procedures and the acceptance criteria used to validate the assay have been described in a number of publications [10,22,23].

2.6. Method comparison

For the experimental details of confocal microscopy, Western blotting and flow cytometry, please see the Supplementary material.

3. Results and discussion

3.1. Characterization of (phospho)peptides

In general, the most critical step in the establishment of a targeted proteomics assay is the selection of proteolytic peptides that represent a candidate protein and could provide specificity and an adequate response. As described in our previous work [20,24], a LC/MS/MS analysis with a list of MRM transition pairs based on either in silico prediction or spectral evidence from public repositories was normally performed to identify the peptide with the greatest abundance [15,25,26]. However, there are some differences between this study and the previous ones. For the site-specific quantification of protein phosphorylation, the levels of both phosphorylated and non-phosphorylated peptides containing desired sites need to be measured. Thus, the abundance of these peptides is of particular importance. Fortunately, the tryptic peptides with Ser15 and Ser82 (13GPSWDPFR20 and 80QLSSGVSEIR89) provided sufficient intensity in MRM. The most abundant forms were their doubly charged ions. Their product ion spectra and LC/MS/MS chromatograms are shown in Fig. 1A. The characteristic sequence-specific b ions and y ions were indicative of peptides. In addition, their corresponding phosphorylated forms (13GPpSWDPFR20 and 80QLpSSGVSEIR89) can be also detected. They were retained longer on the column than their unmodified counterparts (Fig. 1B). Consistent with previous observation, sequence-specific product ions could not be detected or weak in tandem mass spectra due to low abundance of phosphopeptides and unfavored fragmentation [27,28]. Furthermore, specificity of these peptides was checked using a BLAST search. Their sequences were found to be unique to HSP27 (accession no. P04792 (HSPB1_HUMAN)).

On the contrary, the peak for peptide containing Ser78 (76ALSR79) was not easily distinguished in LC/MS/MS. This peptide was very small and hydrophilic. Thus, it was not retained well on commonly used C18 column [29]. In addition, the BLAST result indicated that 76ALSR79 was too short to be unique to HSP27 and could not be used for protein quantification. Under such circumstances, trypsin (the most commonly used enzyme [30]) may not be suitable for protein digestion and another enzymes or multiple enzymes that can better generate specific peptides may be chosen. For instance, a larger peptide (65SPAVAAPAYSRALSRQLSSGVSE87) was obtained after the use of Glutamic-C endopeptidase (Glu-C). However, the occurrence of isobaric phosphopeptides (i.e., phosphopeptides with different phosphorylation sites and the same mass; the peptide was phosphorylated at either Ser78 or Ser82 in this study) emerged as a new issue. This type of phosphopeptides usually has the similar chromatographic and mass spectral behavior and is not easily distinguished from each other. Therefore, stoichiometric quantification of HSP27 phosphorylation at Ser78 will not be discussed in this study. Indeed, we have developed a novel algorithm to simultaneously quantify the isobaric phosphopeptides (draft in preparation).

Then, (phospho)peptides were synthesized for each sequence. The corresponding stable isotope-labeled peptides were also prepared to serve as internal standards. In detail, stable isotope-labeled [D_8]Val and [$^{13}C_9$]Phe were coupled to 80QLSSGVSEIR89 and 13GPSWDPFR20, respectively.

3.2. Development and validation of a LC/MS/MS-based targeted proteomics assay

To provide accurate and precise amount of proteins, the completeness of trypsin digestion must be carefully assessed. However, this issue will not be extensively discussed here. Following the similar process given in our previous work [20,24] and using the substrate peptides containing the same sequence as the (phospho)peptides (to mimic a piece of the targeted protein), the digestion efficiency was calculated by comparing the response ratios of the tryptic peptide after digestion and the equimolar synthetic peptide standard in the digestion. The estimated values were 96.2% and 93.4% (13GPSWDPFR20 and 13GPpSWDPFR20), and 96.9% and 89.1% (80QLSSGVSEIR89 and 80QLpSSGVSEIR89).

Another important step in protein quantification is to generate high-quality MRM [16]. In this study, the transitions that gave the best signal-to-noise and limit of quantification (LOQ) for 13GPSWDPFR20, 13GPpSWDPFR20 and 80QLSSGVSEIR89, 80QLpSSGVSEIR89 were afforded by the product ions y3 *m/z* 419.2, y3 *m/z* 419.2, y2 *m/z* 288.2, and y8 *m/z* 914.6, respectively. These characteristic mass patterns were also observed using the stable isotope-labeled internal standards.

Using the transitions of *m/z* 481.2 → 419.2, *m/z* 485.9 → 419.2 (IS), *m/z* 521.2 → 419.2, 525.9 → 419.2 (IS), *m/z* 538.2 → 288.2, *m/z* 542.4 → 288.2 (IS), *m/z* 578.2 → 914.6, *m/z* 582.4 → 922.5 (IS), a HSP27 LC/MS/MS assay was developed and validated. Solid phase extraction was selected as the technique of choice for sample cleanup and enrichment in this study because, as previously reported, it has shown great promise for sample preparation [24]. Multi-peptide calibration standards were prepared to shorten the analysis time [31]. The calibration curves were constructed using a weighted linear regression model with a weighting factor of $1/x^2$. The relative peak area ratio of the analyte and the stable isotope-labeled internal standard were plotted against concentration. Representative calibration curves are shown in Fig. 1S. The LOQs were 100 ng/mL for non-phosphorylated and 10 ng/mL for phosphorylated peptides. Representative chromatograms of the LLOQ are shown in Fig. 2S. No significant interfering peak was found at the retention time of peptides in the chromatogram of the blank matrix, in agreement with that the analyte response at the LLOQ was at least 5 times the response compared to blank response [32]. To further examine the specificity of MRM transitions for quantification, a second product ion of (phospho)peptides was evaluated here to serve as a confirmation [33,34]. The values determined for pairs of MRM transitions of each peptide were consistent throughout the calibration range (data not shown).

The precision and accuracy of the assay were assessed by observing the response of the QC samples with four different concentrations of (phospho)peptides in three validation runs. The intra- and inter-day precisions were expressed as the percent coefficient of variation (%CV). The accuracy was

obtained by comparing the averaged calculated concentrations to their nominal values (%bias). The results are listed in Table 1S. Both accuracy and precision were ≤±15% (LLOQ, ≤± 20%) [32].

Three cycle freeze–thaw, 48 h post-preparative (4 °C) and 12 h room temperature stabilities were also conducted here. The results indicated that the stability of the peptide was acceptable (data not shown).

3.3. Quantitative time-course analysis of HSP27 and P-gp

Using the LC/MS/MS-based targeted proteomics assay, the level of HSP27 was accurately quantified to be 1.61 pg/cell (~59.6 amol/cell) in MCF-10A cells, 11.38 pg/cell (~421 amol/cell) in MCF-7/WT and 3.47 pg/cell (~129 amol/cell) in MCF-7/ADR cells. Correspondingly, the IC50 (the half maximal inhibitory concentration) values of DOX in these cells calculated

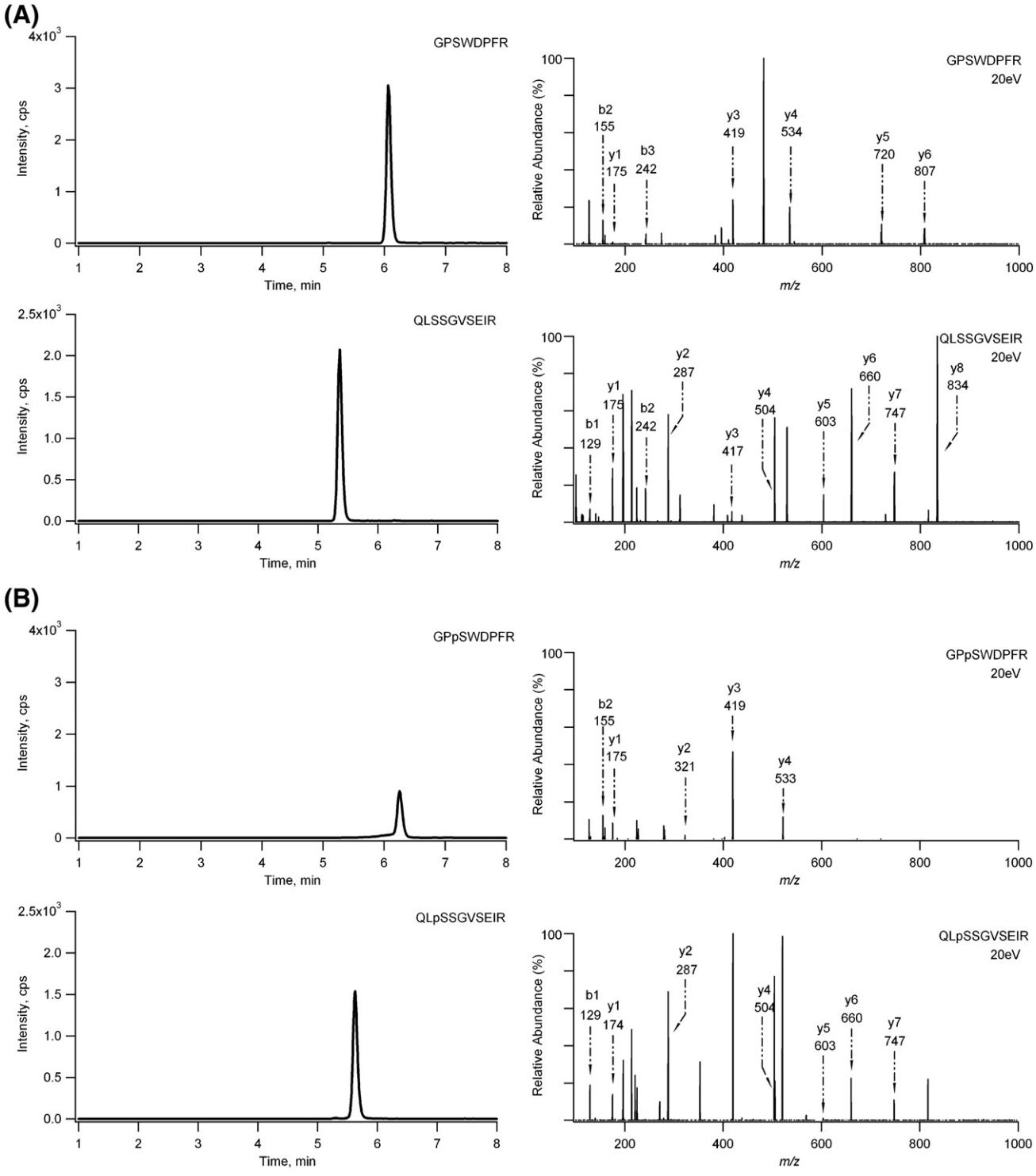

Fig. 1 – (A) Extracted ion chromatograms and product ion spectra of doubly charged ion of 13GPSWDPFR20, 13GPpSWDPFR20, 80QLSSGVSEIR89 and 80QLpSSGVSEIR89.

from the dose–log response curves were 278.2 ± 22.6 nM, 569.7 ± 30.4 nM and 35.6 ± 2.4 μM, respectively. The resistance index (RI) of MCF-7/ADR cells relative to the parental MCF-7/WT cells was 62.5 [35]. Combined with the P-gp data previously described in our work [20], this result supported the previous findings of HSP27 in the growth and P-gp mediated MDR acquisition of cancer cells using antibody-based techniques [3,7]. Compared with the qualitative/semi-quantitative results

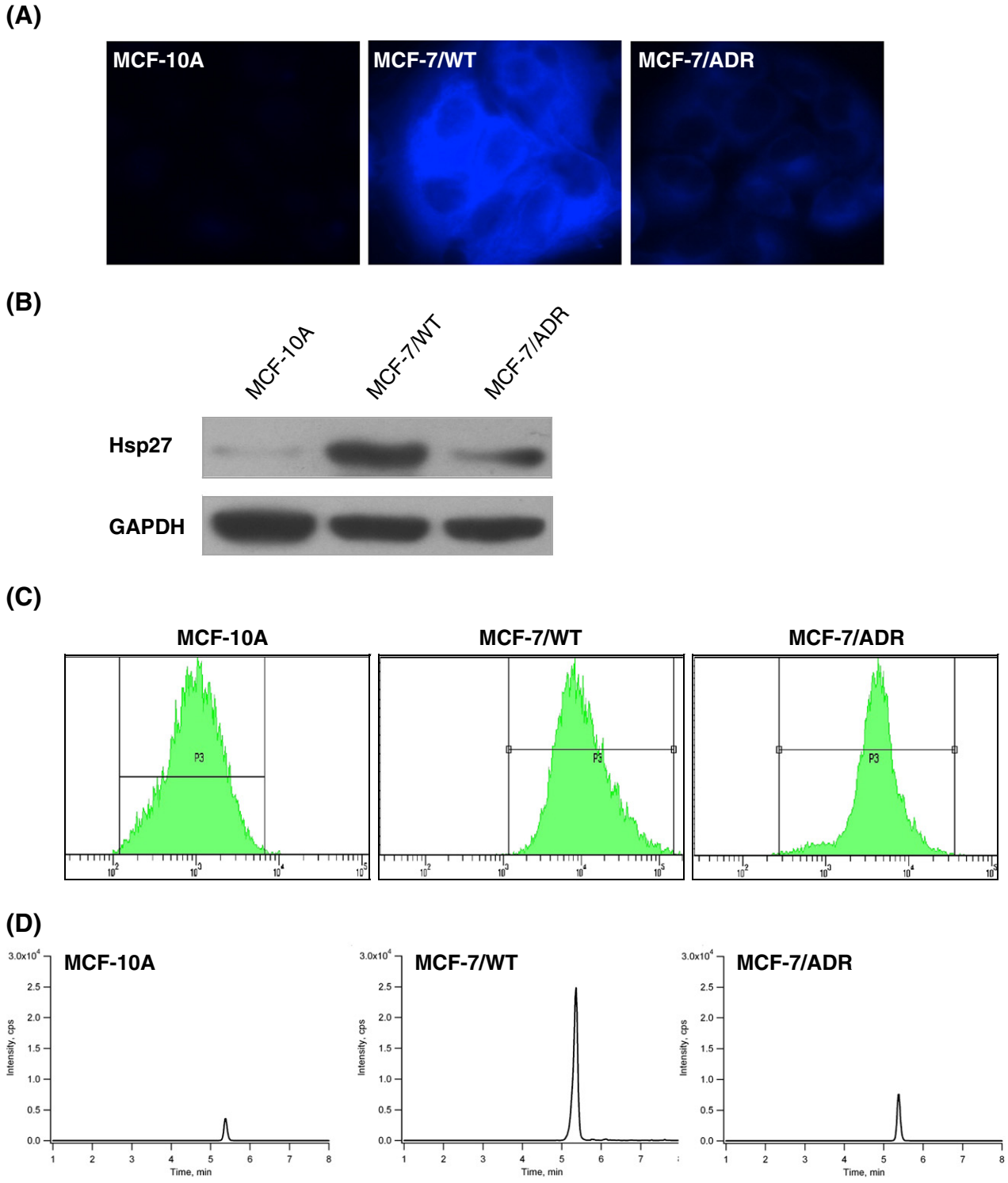

Fig. 2 – Representative images of confocal microscopy, Western blotting, flow cytometry and LC/MS/MS-based targeted proteomics. (A) Confocal images of HSP27 after staining with the Dylight 405 Affinipure Goat Anti-Rabbit IgG. (B) Western blotting of HSP27 expression in the MCF-10A, MCF-7/WT and MCF-7/ADR, normalized with GAPDH. Lane 1: non-tumoral MCF-10A cells; Lane 2: drug-sensitive MCF-7/WT cells; Lane 3: drug-resistant MCF-7/ADR cells. (C) Expression level of HSP27 measured by flow cytometry. (D) LC/MS/MS chromatograms of HSP27.

of confocal microscopy, Western blotting and flow cytometry (Fig. 2), targeted proteomics approach detected the overexpression of HSP27 in MCF-7/WT cells as well as its decrease in MCF-7/ADR cells in values.

In addition to the quantity of various proteins in biological samples, the important information that LC/MS/MS-based targeted proteomics can afford is whether a given protein component of a signaling cascade has gone up or down in response to extracellular stimuli, which is essential for us to understand the cell events in a more accurate context of biological system. In this study, this capability was implemented to closely monitor the HSP27 changes in the development of P-gp mediated MDR. MCF-7/WT cells were cultured for several passages in the presence of 100 ng/mL DOX to induce P-gp. The cells were subsequently analyzed for the levels of HSP27 and P-gp at each end of the passage. The results indicated a significant decrease of HSP27 with the treatment of DOX, corresponding to a slight increase in P-gp expression (Fig. 3 and Table 1). As a control, MCF-7/ADR cells were also incubated with DOX using the same procedures. Consistent with the previous finding [3], no significant change of HSP27 was observed up to the third passage. A similar pattern was also found in other breast cell lines (e.g., T47D and T47D/ADR cells, Fig. 3S). These phenomena implied that HSP27 may participate in the P-gp modulation in DOX-treated cells. Since no more signal transduction factors were monitored here, the association between HSP27 and P-gp can not be fully addressed.

Table 1 – Time-course analysis of HSP27 and P-gp levels in MCF-7/WT cells after the treatment of DOX using LC/MS/MS-based targeted proteomics.

Passages after the treatment of DOX	0	1	2	3
HSP27 (pg/cell)	11.4 ± 0.3	9.73 ± 0.55	8.03 ± 0.25	6.21 ± 0.36
P-gp (fg/cell)	3.62 ± 0.20	4.41 ± 0.20	4.74 ± 0.14	5.83 ± 0.28

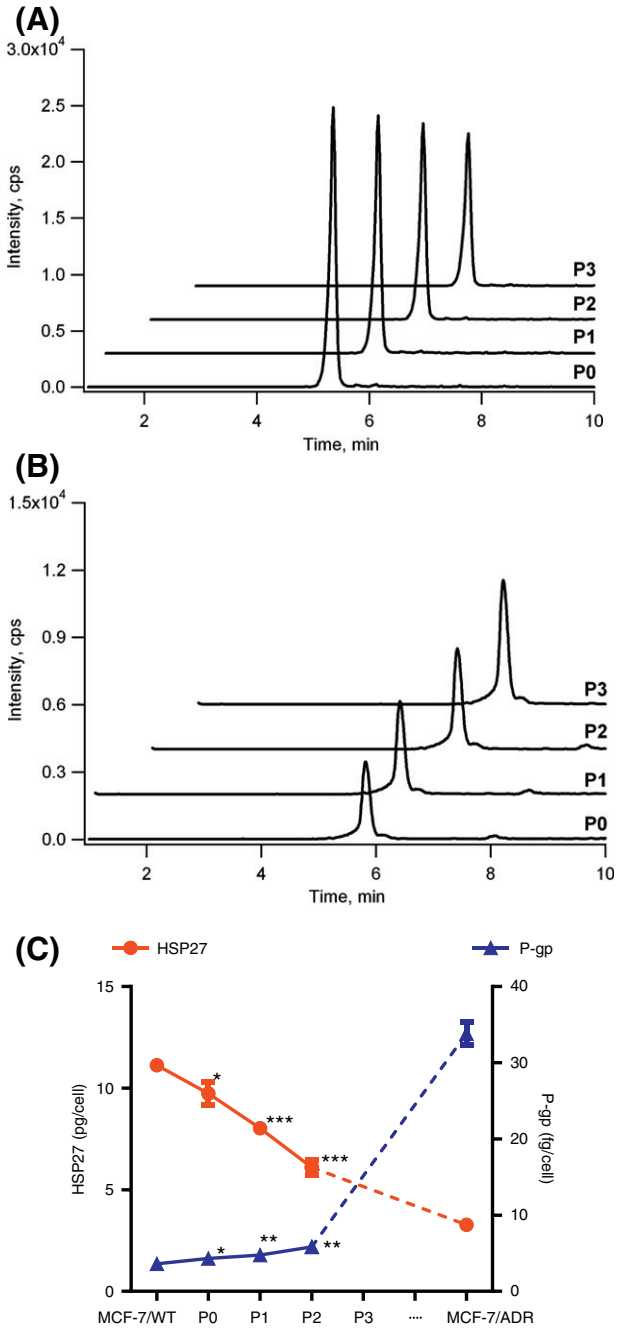

Fig. 3 – The LC/MS/MS chromatograms of HSP27 (A) and P-gp (B) in freshly prepared (P0) and passages 1, 2, and 3 (P1, P2, P3) MCF-7/WT cells after the treatment of DOX. (C) The levels of HSP27 and P-gp in each passage of MCF-7/WT cells with DOX treatment and MCF-7/ADR cells (significant differences compared with control (untreated) are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

Notably, a variety of pathways have been suggested for cancer cells to acquire drug resistance as described previously [3]. Other than the increased membrane accumulation of various ATP-binding cassette (ABC) transporters, another major pathway is the direct suppression of apoptosis. The suppression of apoptotic pathways is most likely due to the accumulation of mutant p53 (mutp53) with "gain of function" and to the increased expression of antiapoptotic proteins, which HSP27 may also interact with [36]. Thus, we can not exclude the possibility that the variations of the expression levels of two proteins may be just a chance happening between two cellular events (e.g., DOX induced P-gp mediated drug efflux and HSP27 regulated apoptosis). More experiments by simultaneously monitoring more than one pathway may be further required for better elucidation. Nevertheless, this study provided the evidence that targeted proteomics approach can be applied to study cellular events quantitatively.

3.4. Stoichiometric quantification of HSP27 phosphorylation

Protein phosphorylation is one of the most important approaches to regulate cellular functions. The regulatory information is usually from specific phosphorylation site. Thus, determination of site-specific phosphorylation stoichiometry and quantification of the extent to which phosphorylation has changed is of importance. As pointed out earlier, the major issue with current antibody-based methods is the lack of quantitative capability. This disadvantage is more pronounced in the determination of phosphorylation

Table 2 – The stoichiometry of HSP27 phosphorylation at Ser15 and Ser82 in MCF-7/WT and MCF-7/ADR cells w/o DOX using LC/MS/MS-based targeted proteomics.

	Protein amounts (pg/cell)			Phosphorylation stoichiometry (%)	
	HSP27	pSer15	pSer82	pSer15	pSer82
MCF-7/WT	11.4 ± 0.3	$(1.42 \pm 0.05) \times 10^{-1}$	$(1.53 \pm 0.08) \times 10^{-1}$	1.21 ± 0.05	1.33 ± 0.08
MCF-7/ADR	3.47 ± 0.16	$(4.96 \pm 0.26) \times 10^{-2}$	$(6.31 \pm 0.24) \times 10^{-2}$	1.43 ± 0.10	1.82 ± 0.08
p values	<0.001	<0.001	<0.001	<0.05	<0.01

stoichiometry. Currently, a majority of studies reported phosphorylation events that failed to distinguish changes in phosphorylation from protein expression [24]. Nearly 25% of what appears to be differential protein phosphorylation was actually due to the changes in protein expression [37]. To better discern protein phosphorylation, each phosphorylation event required the simultaneous quantification of protein in its non-phosphorylated and phosphorylated forms, and then normalization. Using targeted proteomics approach, we can better achieve this goal. Since induced HSP27 phosphorylation has been associated with the drug resistance acquisition [9,38], the stoichiometric quantification of HSP27 phosphorylation at Ser15 and Ser82 was attempted in this study (Table 2). As a result, an increase of phosphorylation was shown in MCF-7/ADR cells, and the increase extent was greater at Ser82. In comparison, this shift could not be easily distinguished in Western blotting using multiple phospho-specific antibodies (Fig. 4S) [39]. Subsequent time course analysis indicated that HSP27 phosphorylation at Ser82 was rapidly induced and peaked within the first 30 min after the incubation with DOX (Fig. 4A). In comparison, no significant change of phosphorylated HSP27 was observed in MCF-7/ADR cells. This phenomenon was consistent with the 'early' p38-dependent HSP27 phosphorylation as previously reported [40]. To further confirm this thought, an inhibition of HSP27 phosphorylation was performed using SB203580 (Selleck Chemicals, Houston, TX, USA). SB203580 is a pyridinyl imidazole inhibitor of p38 MAPK activation, while HSP27 phosphorylation is mediated by p38 MAPK [21,41]. As shown in Fig. 4B, pretreatment with SB203580 blocked DOX induction and HSP27 phosphorylation effectively. These results suggested a site-specific phosphorylation of HSP27, which may possibly lead to downstream cellular events such as P-gp mediated MDR.

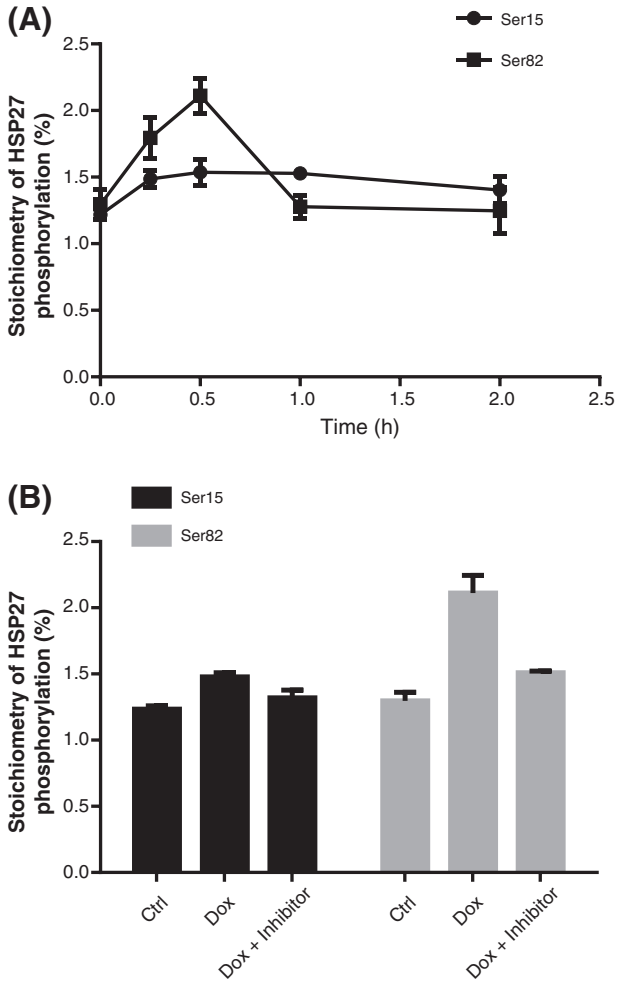

Fig. 4 – Time-dependent stoichiometry of HSP27 phosphorylation at Ser15 and Ser82 in MCF-7/WT cells with the incubation of DOX (A), and the effect of inhibitor SB203580 on HSP27 phosphorylation in MCF-7/WT cells treated with DOX for 30 min (B).

4. Conclusions

In this study, the protein level and site-specific phosphorylation stoichiometry of HSP27 were determined in breast cells using a simple and sensitive LC/MS/MS-based targeted proteomics assay. Consistent with the previous findings regarding HSP27 in P-gp mediated MDR acquisition, the change of protein level and phosphorylation extent was observed and monitored in a time course. Compared to the conventional analytical methods that provided only the semi-quantitative values, targeted proteomics approach provided a quantitative mean to study time-dependent cellular events in terms of biological significance. However, these advantages of targeted proteomics need further elucidation using more complex protein systems, for instance, multi-proteins with multi-phosphorylated forms in more than one signal transduction pathways.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.05.016.

Transparency document

Transparency Document associated with this article can be found, in the online version.

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