

A targeted proteomics approach to the quantitative analysis of ERK/Bcl-2-mediated anti-apoptosis and multi-drug resistance in breast cancer

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Received: 29 April 2016 / Revised: 26 July 2016 / Accepted: 28 July 2016 / Published online: 10 August 2016
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Abstract Apoptosis suppression caused by overexpression of anti-apoptotic proteins is a central factor to the acquisition of multi-drug resistance (MDR) in breast cancer. As a highly conserved anti-apoptotic protein, Bcl-2 can initiate an anti-apoptosis response via an ERK1/2-mediated pathway. However, the details therein are still far from completely understood and a quantitative description of the associated proteins in the biological context may provide more insights into this process. Following our previous attempts in the quantitative analysis of MDR mechanisms, liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based targeted proteomics was continually employed here to describe ERK/Bcl-2-mediated anti-apoptosis. A targeted proteomics assay was developed and validated first for the simultaneous quantification of ERK1/2 and Bcl-2. In particular, ERK isoforms (i.e., ERK1 and ERK2) and their differential phosphorylated forms including isobaric ones were distinguished. Using this assay, differential protein levels and site-specific phosphorylation stoichiometry were observed in parental drug-sensitive MCF-7/WT cancer cells and drug-resistant MCF-7/ADR cancer cells and breast tissue samples from two groups of patients who were either suspected or diagnosed to have drug resistance. In addition, quantitative analysis of the time course of

both ERK1/2 and Bcl-2 in doxorubicin (DOX)-treated MCF-7/WT cells confirmed these findings. Overall, we propose that targeted proteomics can be used generally to resolve more complex cellular events.

Keywords Extracellular signal-regulated protein kinase (ERK) · Bcl-2 · Anti-apoptosis · Multi-drug resistance (MDR) · Isoforms · Targeted proteomics

Introduction

Multi-drug resistance (MDR) represents a major challenge in breast cancer chemotherapy. So far, several mechanisms have been reported for MDR acquisition [1]. Other than drug efflux we previously investigated [2, 3], apoptosis suppression caused by overexpression of anti-apoptotic proteins is a central factor to the acquisition of MDR in breast cancer. One of the most frequently proposed factors in apoptosis suppression is an enhanced expression of anti-apoptotic proteins, such as Bcl-2 [4]. Bcl-2 is a highly conserved and important regulator of apoptosis [5]. Its overexpression can attenuate cell death induced by most chemotherapy drugs and thus has been associated with MDR in a wide range of cancers, including breast cancer [6]. To date, the enhancement of Bcl-2 has been observed in 70 % of clinical breast cancer [7]. Even though the precise molecular mechanism of Bcl-2 remains far from completely understood, several kinases have been proposed to be responsible for its activation, one of which is extracellular signal-regulated kinase (ERK) [8].

The ERK family consists of two closely related mitogen-activated protein kinases (MAPKs), ERK1 (p44, MAPK3), and ERK2 (p42, MAPK1). Accumulating evidence suggests that ERK1 and ERK2 may play distinct functions [9, 10], leading to the importance of discerning the effect of individual ERK

Electronic supplementary material The online version of this article (doi:10.1007/s00216-016-9847-7) contains supplementary material, which is available to authorized users.

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isoforms in the cellular events. In addition, ERK1/2 needs to be activated by phosphorylation and several phosphorylation sites have been found in the activated ERK1/2. Among them, two key regulatory sites are the neighboring threonine and tyrosine (T202 and Y204 in ERK1, and T185 and Y187 in ERK2) [11]. Generally, dual phosphorylation is required to fully activate ERK1/2; however, partially phosphorylated forms exist [12]. There is evidence implying that the activated mono-phosphorylated forms of ERK1/2 may be more closely related to drug resistance [13]. Overall, anti-apoptosis response may be initiated through the upregulation of Bcl-2 via an ERK1/2-mediated pathway. Thus, a more accurate description of ERK/Bcl-2-mediated anti-apoptosis may provide significant insights into MDR acquisition in breast cancer.

During addressing the signal transduction mechanisms, essential information that the researchers can rarely obtain is the exact amounts of proteins in biological samples. At present, antibody-based methods have been widely employed to measure the level of proteins. These methods offer important information on protein levels, whereas they frequently fail to provide adequate reproducibility and specificity [14]. Furthermore, it is not easy to interpret the results due to the fact that proteins may be present in several forms, such as phosphorylated and non-phosphorylated forms [15], especially those phosphorylated forms with different phosphorylation sites but the same mass (i.e., isobaric forms [16, 17]). Moreover, most antibody-based assays suffer from inadequate availability and poor quality of phospho-specific antibodies [18]. The last limitation of antibody-based assays is owing to their qualitative or semi-quantitative nature [19]. As an alternative approach, liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based targeted proteomics is a technology for detecting proteins of interest with high sensitivity, quantitative accuracy, and reproducibility [20]. The key concept of the targeted analysis is to specifically explore the proteins at the peptide level [21, 22]. Surrogate peptides are produced via enzymatic digestion of the target protein. Selected/multiple reaction monitoring (SRM/MRM) is utilized to monitor the selected surrogate peptide [23]. In this way, multiple proteins and associated protein modification can be analyzed in one experiment.

In this work, we utilized the quantitative feature of targeted proteomics to investigate ERK/Bcl-2-mediated anti-apoptosis and MDR acquisition in the biological context. A LC-MS/MS-based targeted proteomics assay for the simultaneous quantification of ERK1/2 (i.e., non-phosphorylated, isobaric mono-phosphorylated, and di-phosphorylated forms) and Bcl-2 was first developed and validated. This assay was then applied to the quantitative monitoring of these protein forms in parental drug-sensitive MCF-7/WT cancer cells, drug-resistant MCF-7/ADR cancer cells, and breast tissue samples from two groups of patients who were either suspected or diagnosed to have drug resistance (36 patients in each group).

Drug resistance was induced in MCF-7/WT cells by incubating the cells with doxorubicin (DOX). In the presence of DOX, the association of ERK1/2 protein level and site-specific phosphorylation stoichiometry with Bcl-2 was detected in a time course. Finally, we compared the outcome with that obtained using conventional analytical methods including fluorescence microscopy and Western blotting.

Materials and methods

Chemicals and reagents

Peptides and phosphopeptides were provided by ChinaPeptides Co., Ltd. (Shanghai, China). The stable isotope-labeled amino acids were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and applied in the synthesis of internal standards (ChinaPeptides Co., Ltd., Shanghai, China). The purities of the peptides were provided by the manufacturer. Sequencing grade modified trypsin was obtained from Promega (Madison, WI, USA). DL-dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate (NH_4HCO_3), and Tris-HCl were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid disodium salt ($\text{EDTA}2\text{Na}$), formic acid (FA), and trifluoroacetic acid (TFA) were supplied by Aladdin Chemistry Co., Ltd. (Shanghai, China). Methanol and acetonitrile (ACN) were purchased from Tedia, Inc. (Fairfield, OH, USA). Fetal bovine serum (FBS), Dulbecco's modified Eagle media (DMEM), and penicillin-streptomycin solution were obtained from Thermo Scientific HyClone (Logan, UT, USA). Trypan blue and sodium dodecyl sulfate (SDS) were obtained from Generay Biotech Co., Ltd. (Shanghai, China). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA).

Preparation of stock solutions, calibration standards, and quality controls

The peptides (phosphopeptides) were weighted accurately and dissolved in deionized water to give a final concentration of 1 mg/mL. All the solutions were stored in the dark at -20°C . An isotope-labeled peptide (non-phosphorylated form) was used as a single internal standard. The selection of a suitable internal standard will be described below. In a similar manner, an internal standard stock solution at 5 $\mu\text{g}/\text{mL}$ was prepared. Then, this stock solution was diluted with an ACN:water mixture (50:50, v/v) containing 0.1 % FA to give a final concentration of 500 ng/mL.

Herein, serial dilution of the stock solution was employed to prepare calibration standards in a cellular extract depleted of target protein as the matrix. The experimental details about immuno-depletion of cellular extract are provided in the Electronic Supplementary

Material (ESM). Generally, non-phosphorylated peptides were combined in proportions to create the calibration standards that contain 100, 200, 500, 1000, 2500, 5000, and 10,000 ng/mL of each peptide. Similarly, the concentrations of phosphorylated peptides in the calibration standards were 10, 20, 50, 100, 250, 500, and 1000 ng/mL for each peptide in a mixture. The quality controls (QC) standards (i.e., lower limit of quantification (LLOQ), low QC (LQC), mid QC (MQC), and high QC (HQC)) 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. Notably, the calibration and QC standards of Bcl-2 were prepared in the similar manner of phosphopeptides, due to the low cellular abundance of protein.

Cell culture, tissue collection, and immunoprecipitation of target proteins

MCF-7/WT cells (ATTC, Manassas, VA) and MCF-7/ADR cells (Keygen Biotech, Nanjing, China) were cultured in DMEM media supplemented with 10 % fetal bovine serum and 1 % penicillin/streptomycin at 37 °C under a 5 % CO₂ atmosphere. The cells were lifted using 0.25 % trypsin and seeded at a density of 0.5×10^6 cells per 100 mm plate. The medium was changed every other day for 5–7 days. 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 [24]. Experiments were performed using the cells incubated without DOX for 48 h. Cell viability was assessed by trypan blue (0.4 %) exclusion and the viable cell number was counted with a hemocytometer (Qiujing, Shanghai, China). Two individual counters each counted the cells three times.

Cells ($\sim 10^7$ cells) were washed twice with ice-cold PBS and then lysed in 400 μ L of RIPA lysis buffer (Beyotime Institute of Biotechnology, China) containing 1 % protease inhibitor cocktail (Sigma-Aldrich, MO, USA) and 1 % phosphatase inhibitor cocktail (Sigma-Aldrich, MO, USA). Afterward, the sample was incubated on ice for 45 min, followed by centrifugation at $16,000 \times g$ for 10 min and split into two fractions. Half was used for immunoprecipitation of ERK1/2, and the other half was for Bcl-2. In detail, mouse monoclonal anti-ERK1/2 antibody (Abcam, Cambridge, UK) or mouse monoclonal anti-Bcl-2 antibody (Proteintech, Chicago, IL, USA) bound to protein A/G Agarose (Abmart, Shanghai, China) was added to the cell lysate. Then, nonspecifically bound proteins were removed by affinity purification and captured target protein was eluted from the beads by heating at 95 °C for 5 min and with a 1 % SDS solution. The protein concentration was determined using a BCA protein assay kit (Beyotime, Jiang Su, China) according to the manufacturer's protocol.

Breast tissue collection in the present study was approved by the Institutional Review Board of Nanjing Medical University and obtained with informed consent from patients at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) consecutively between January 2013 and February 2015. The patients were biologically unrelated, but all were of Han Chinese ethnicity. Paired breast tumor tissue and adjacent normal tissue samples were from two groups of patients who had invasive breast cancer and were either suspected or diagnosed to have drug resistance (36 patients in each group; 51.6 ± 7.4 years (35–61 years) for group *w/o* drug resistance and 52.1 ± 6.8 years (35–60 years) for group *w* drug resistance). For those patients with suspected drug resistance, initial active chemotherapy was evaluated by reduced tumor size, decreased serological tumor markers, and improved symptoms. However, after a variable period, the tumor not only was resistant to the agents treated but also continued to develop resistance to combinations of non-cross-resistant regimens [25]. Tissue samples were confirmed as cancerous or normal by hospital's pathologist and then stored frozen at -80 °C until analysis. After thawing at room temperature, tissue samples were rinsed thoroughly with deionized water for protein extraction. Following removal of fat tissue, the remaining tissue was cut into small pieces and transferred to tubes. Approximately 50 mg tissue was weighted and resuspended in RIPA lysis buffer containing 1 % protease inhibitor cocktail and 1 % phosphatase inhibitor cocktail. A Bio-Gen PRO200 homogenizer (PRO Scientific Inc., Oxford, CT, USA) was used to homogenize samples. After centrifugation, the collected samples were treated with RIPA lysis buffer and extracted using the procedure described above.

In-solution tryptic digestion

50 μ L of 50 mM NH₄HCO₃ was applied to mix with a quantity of 100 μ L of each sample. Subsequently, 50 mM DTT was added to reduce the protein with a final concentration of 10 mM, followed by incubation at 60 °C for 20 min. 400 mM IAA was added to alkylate the sample with a final concentration of 50 mM at room temperature for 6 h in the dark. The sample was then incubated at 37 °C for 24 h with an addition of sequencing grade trypsin. 10 μ L of 0.1 % TFA was added to stop the reaction. Afterward, the tryptic peptide sample was mixed with 100 μ L of the internal standard solution and transferred 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. 2 mL of water and 2 mL of ACN:water (50:50, *v/v*) was applied to wash the cartridge, followed by elution with 1 mL of 100 % ACN.

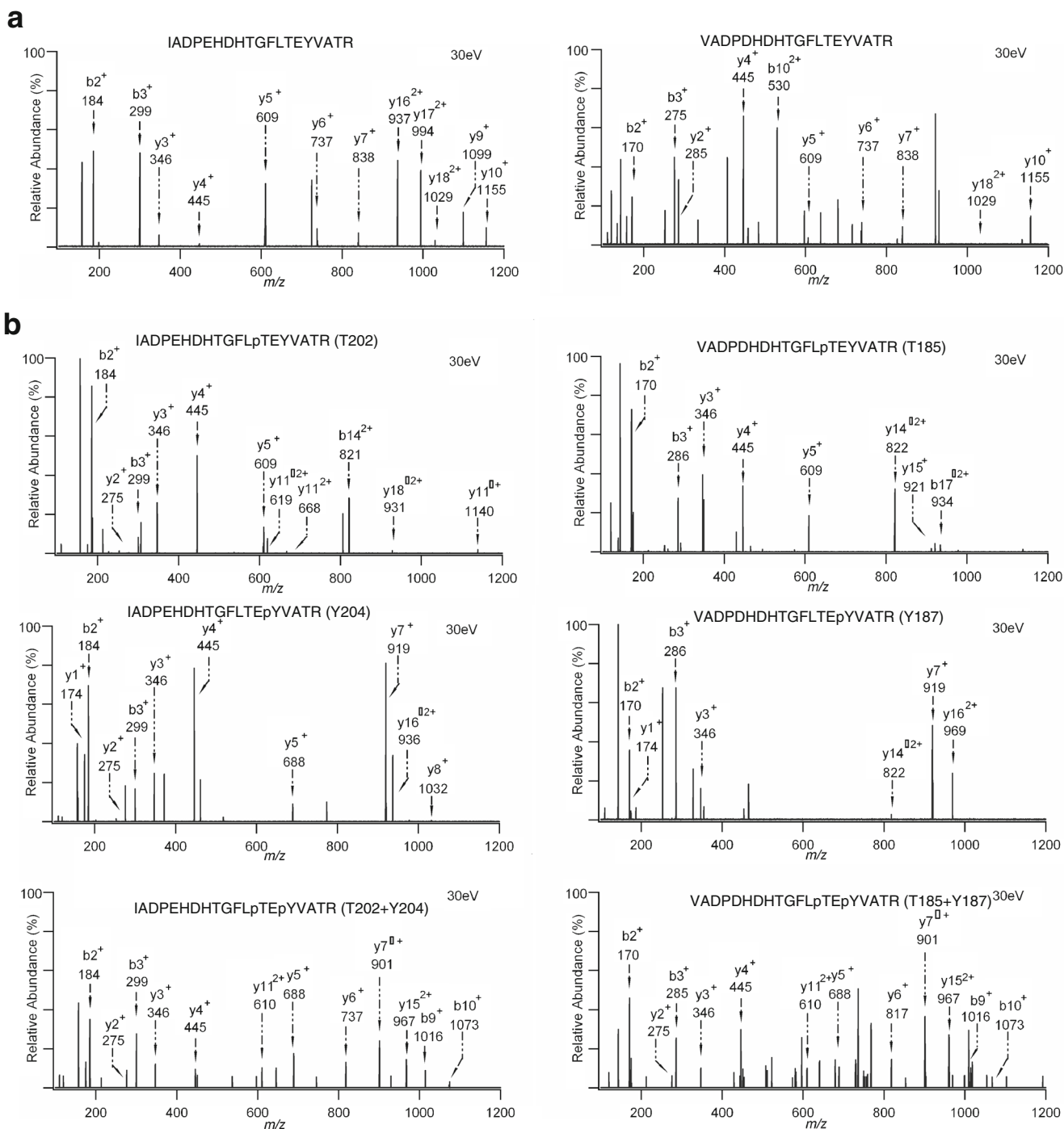


Fig. 1 a, b Product ion spectra of triply charged ions of 190IADPEHDHTGFLTEYVATR208, T202, Y204, and T202 + Y204 of ERK1 and 173VADPDHDHTGFLTEYVATR191, T185, Y187, and T185 + Y187 of ERK2. *Square* = -98 Da ($-\text{H}_3\text{PO}_4$ or $-(\text{H}_2\text{O} + \text{HPO}_3)$) or -80 Da ($-\text{HPO}_3$)

Finally, the sample was dried and resuspended in 100 μL of ACN:water (50:50, v/v) containing 0.1 % FA.

LC-MS/MS assay development and validation

Quantification was performed on an Agilent Series 1200 HPLC system (Agilent Technologies, Waldbronn,

Germany) coupled with a 6410 Triple Quad LC/MS mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

The liquid chromatography separations were performed on a hypersil gold column (3 μm , 20 mm \times 2.1 mm; Thermo Fisher Scientific, USA) at room temperature. The mobile phase consisted of solvent A (0.1 % FA in

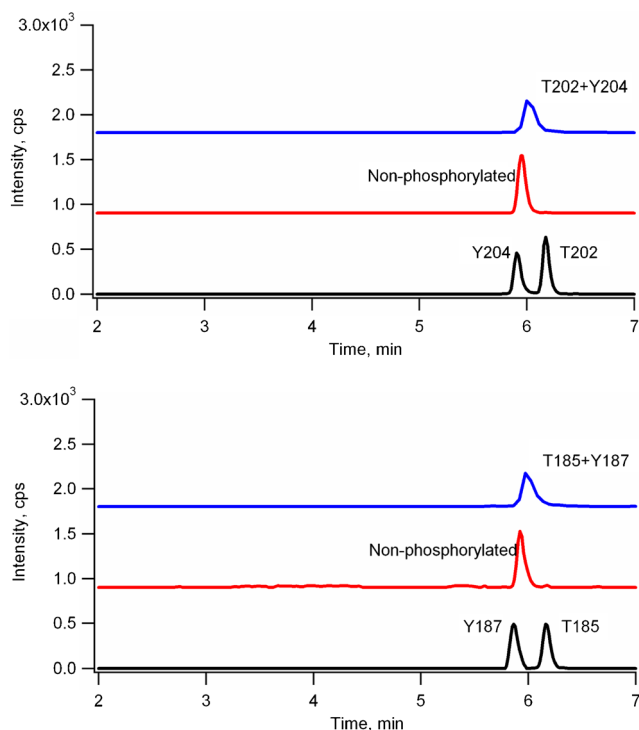


Fig. 2 Extracted ion chromatograms of triply charged ions of 190IADPEHDHTGFLTEYVATR208, T202, Y204, and T202 + Y204 of ERK1 and 173VADPDHDHTGFLTEYVATR191, T185, Y187, and T185 + Y187 of ERK2

water) and solvent B (0.1 % FA in methanol). A linear gradient at a flow rate of 0.3 mL/min was applied with the following proportions of solvent B: 10 % (0 min) → 10 % (1 min) → 90 % (4 min) → 90 % (8 min) → 10 % (9 min). The injection volume of samples was set at 10 μ L.

The mass spectrometer was equipped with an electrospray ion source. The peptides were detected in the positive MRM mode. The parameter settings were Q1 and Q3 at unit resolution, capillary voltage at 4000 V, nebulizer pressure at 35 psi, drying gas temperature at 350 $^{\circ}$ C, and drying gas flow at 10 L/min. Data analysis was performed using Agilent MassHunter Workstation Software (version B.01.03).

Table 1 Characterization of surrogate peptides of ERK1/2 and Bcl-2

Peptides	Digestion efficiency	Abundant Product Ions (m/z)	MRM transitions
190IADPEHDHTGFLTEYVATR208 (ERK1)			
Non-phosphorylated	96.2 %	y4	724.9 → 446.0
T202, Y204	95.9 %, 95.2 %	y3	751.6 → 346.7
T202 + Y204	94.1 %	y4	777.9 → 446.0
173VADPDHDHTGFLTEYVATR191 (ERK2)			
Non-phosphorylated	96.4 %	y5	715.4 → 609.0
T185, Y187	95.4 %, 95.3 %	y3	742.1 → 346.7
T185 + Y187	94.5 %	y5	768.6 → 688.9
130FATVVEELFR139 (Bcl-2)	95.3 %	y1	605.8 → 173.6

The assay was validated for accuracy, precision, linearity, limit of quantification (LOQ), and stability. The detailed procedures and acceptance criteria utilized to validate the assay have been described in many publications [14, 26, 27]. For each sample, three replicates were made.

Method comparison

Experimental details of Western blotting and fluorescence microscopy are provided in the ESM.

Results and discussion

Characterization of (phospho)peptides

Following the procedure described in our previous work [3, 28–30], the surrogate peptide of Bcl-2 can be easily characterized (130FATVVEELFR139). However, ERK is a more complex protein system with two closely related isoforms containing markedly similar amino acid sequence and differential phosphorylated forms. Thus, we will focus on the (phospho)peptide characterization of ERK1/2 here. Since the major phosphorylation sites (i.e., tyrosine and threonine) of ERK1/2 are present in the same tryptic peptide (190IADPEHDHTGFLTEYVATR208 of ERK1 and 173VADPDHDHTGFLTEYVATR191 of ERK2), the peptide will be detected in several forms (i.e., non-, mono-, and di-phosphorylated peptides). Indeed, mono-phosphorylated peptides with phosphorylation sites at either tyrosine or threonine are isobaric phosphopeptides. To quantitatively measure the site-specific phosphorylation of proteins, several issues need to be addressed. Firstly, the surrogate peptides in both phosphorylated and non-phosphorylated forms should be quantified, respectively. Secondly, the peptide sequence should be unique to the protein analyzed via a BLAST search. The last and most important point that deserves consideration is that isobaric phosphopeptides are not

Table 2 Protein levels and phosphorylation stoichiometry of ERK1/2 and Bcl-2

	Protein amount per cell (pg/cell)		Phosphorylation stoichiometry (%)	
	MCF-7/WT	MCF-7/ADR	MCF-7/WT	MCF-7/ADR
ERK1				
Non-phosphorylated	1.52 ± 0.08	1.73 ± 0.08	–	–
T202	(2.02 ± 0.09) × 10 ⁻²	(7.31 ± 0.14) × 10 ⁻²	1.27 ± 0.09	3.69 ± 0.16
Phosphorylated				
Y204	(1.96 ± 0.12) × 10 ⁻²	(7.12 ± 0.16) × 10 ⁻²	1.23 ± 0.10	3.59 ± 0.17
T202 + Y204	(2.62 ± 0.13) × 10 ⁻²	(10.9 ± 0.4) × 10 ⁻²	1.65 ± 0.12	5.49 ± 0.28
Total	1.59 ± 0.08	1.98 ± 0.08	3.15 ± 0.19	12.8 ± 0.6
ERK2				
Non-phosphorylated	3.28 ± 0.16	3.53 ± 0.18	–	–
T185	(4.07 ± 0.19) × 10 ⁻²	(13.9 ± 0.03) × 10 ⁻²	1.19 ± 0.08	3.48 ± 0.17
Phosphorylated				
Y187	(3.87 ± 0.17) × 10 ⁻²	(14.2 ± 0.37) × 10 ⁻²	1.13 ± 0.07	3.57 ± 0.17
T185 + Y187	(5.56 ± 0.18) × 10 ⁻²	(16.7 ± 0.42) × 10 ⁻²	1.62 ± 0.09	4.19 ± 0.21
Total	3.41 ± 0.16	3.98 ± 0.18	3.92 ± 0.20	11.1 ± 0.6
Bcl-2	(76.6 ± 1.3) × 10 ⁻³	(138.6 ± 2.4) × 10 ⁻³	–	–

easy to be distinguished in LC-MS/MS [31]. Employment of product ions related to the phosphorylation site of interest could be a resolution [32]. However, site-specific fragment ions sometimes cannot be detected or produce weak signals in tandem mass spectra due to a low abundance of phosphopeptides and unfavorable fragmentation [33, 34].

In this study, the most abundant forms of 190IADPEHDHTGFLTEYVATR208 and 173VADPDHDHTGLTEYVATR191 were their triply charged ions. The sequence-specific b ions and y ions confirmed the identity of peptides (Fig. 1a). Then, their corresponding isobaric mono- and di-phosphorylated forms referring to T202, Y204, and T202 + Y204 of ERK1 and T185, Y187, and T185 + Y187 of ERK2 can be also detected. Their product ion spectra are shown in Fig. 1b. Using T202 and Y204 as an example of isobaric phosphopeptides, most observed product ions were common ones. However, there were a few site-specific ions observed, for instance, y₅⁺ m/z 609 for T202 and y₅⁺ m/z 688 for Y204, which was different from our previous observation of HSP27 Ser78 and Ser82 with undetectable site-specific product ions [35]. Alternatively, baseline separations of isobaric phosphopeptides could be achieved after chromatographic optimization (Fig. 2), making it possible to simultaneously detect the isobaric phosphopeptides using their common product ions. Inconsistent with the general behavior of phosphorylated peptides [36], peptide containing a phosphothreonine (e.g., T202) displayed an increased retention time. Notably, even if these isobaric phosphopeptides could not be distinguished by either

chromatographic separation or site-specific product ions, we have previously developed a novel algorithm for this type of quantification in our lab [35].

Peptide specificity was first evaluated using a BLAST search. The sequences of peptides were unique to ERK1, ERK2, and Bcl-2 (accession no. P27361 (MK03_HUMAN), P28482 (MK01_HUMAN), and P10415 (BCL2_HUMAN)). Afterward, synthetic (phospho)peptides were prepared for each sequence. The corresponding stable isotope-labeled peptides were also synthesized to serve as internal standards. As mentioned previously, isotope-labeled synthetic peptides in their non-phosphorylated form were used as single internal standards. Specifically, stable isotope-labeled [¹³C₆]Ile was coupled to 190IADPEHDHTGFLTEYVATR208 at position 1, and [D₈]Val was coupled to 173VADPDHDHTGFLTEYVATR191 at position 1 and 130FATVVEELFR139 at position 4, respectively.

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

Accurate and precise quantification of proteins relies heavily on the completeness of trypsin digestion. This issue has been extensively discussed in our previous targeted proteomics studies [3, 37, 38]. Following the similar approach and using the substrate peptides containing the same sequence as the surrogate peptides, the digestion efficiency was estimated by tryptic digestion of these peptides. The calculated values were in the range of 94.1–96.4 % (Table 1). Another major concern is to create high-quality MRM [39]. In the present study, the transitions that gave the

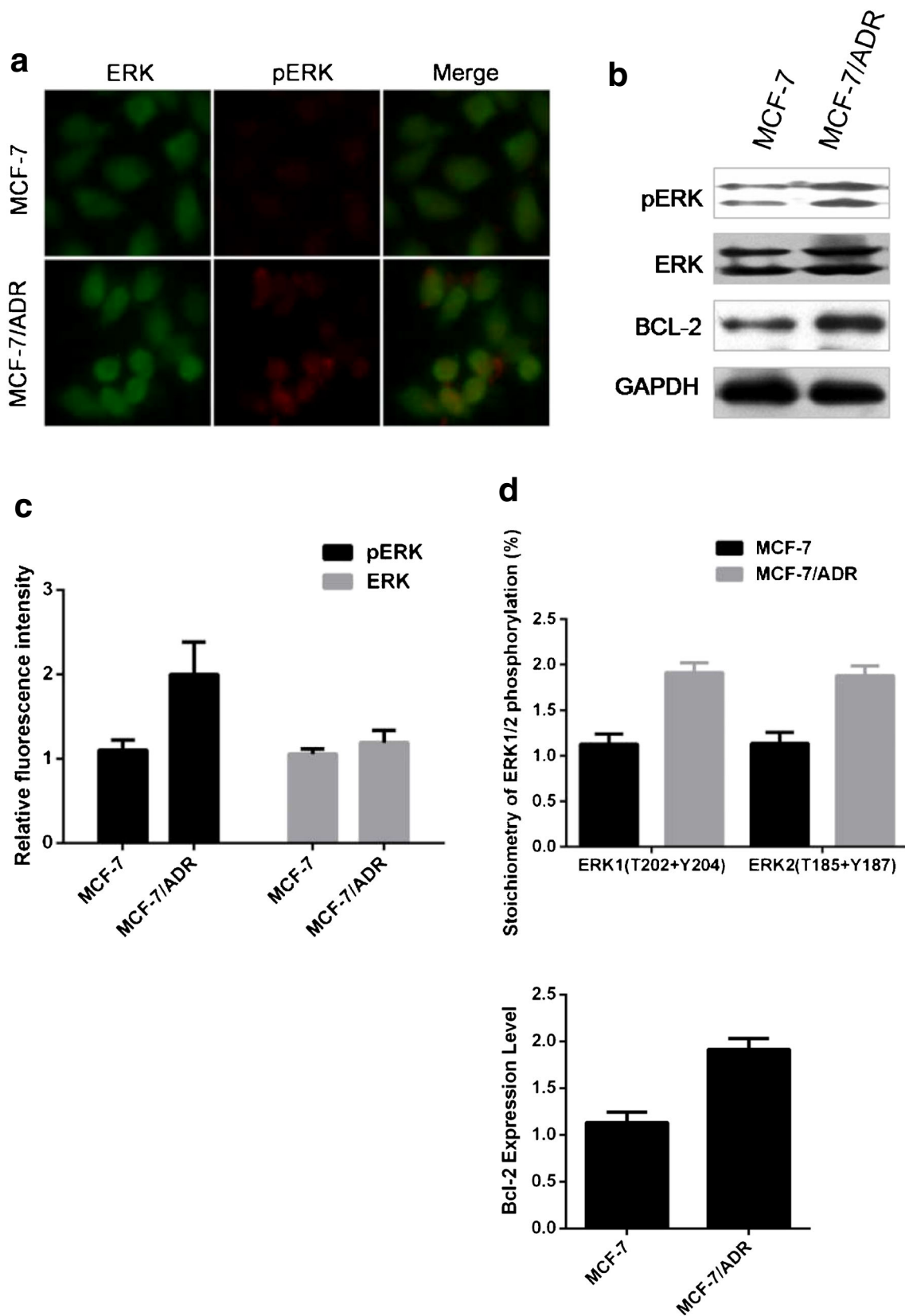


Fig. 3 Representative images of fluorescence microscopy and Western blotting. **a** Fluorescence images of ERK and phosphorylated ERK (pERK) after staining with Dylight 488 Affinipure Goat Anti-Mouse IgG and Dylight 594 Affinipure Goat Anti-Rabbit IgG, respectively. **b**

Western blotting of ERK, pERK, and Bcl-2 expression in the MCF-7/WT and MCF-7/ADR cells, normalized with GAPDH. **c, d** Semi-quantitative analysis of fluorescence microscopy and Western blotting results

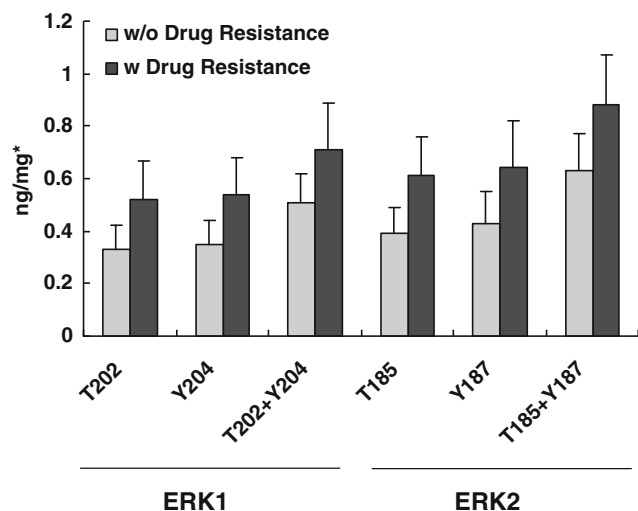


Fig. 4 Differential forms of ERK1/2 phosphorylation in breast tumor tissue samples from two groups of patients who were either suspected or diagnosed to have drug resistance. *ng target protein/mg breast tissue. The values are averaged from 36 patients in each group

best limit of quantification (LOQ) and signal-to-noise ratios were also provided in Table 1. Using these transitions, a LC-MS/MS assay for the quantification of ERK1/2 and Bcl-2 was developed and validated. Prior to analysis, immunoprecipitation was employed to capture target proteins and the features of its combination with LC-MS/MS have been well discussed [40]. Solid-phase extraction (SPE) was the technique of choice for sample cleanup and enrichment in this study, because previous evidence indicates that SPE is a promising technique for sample preparation [41]. Multi-peptide calibration standards effectively reduce the analysis time [42]. The calibration curves for each isomer in the two transitions were regressed using a $1/x^2$ weighting factor. The relative peak area ratio of the analyte and the stable isotope-labeled internal standard was plotted against concentration. Representative calibration curves are shown in Fig. S1 (see ESM). The LOQs were 100 ng/mL for non-phosphorylated and 10 ng/mL for phosphorylated peptides. The result indicated that no significant interfering peak was found at the retention time of the peptides in the chromatograms of the blank matrix (LLOQ response was >5 times the response of the blank matrix) [43]. The specificity of the assay was further confirmed using a second product ion of (phospho)peptides [44, 45]. As a result, the two pairs of MRM transitions of each peptide were in strong agreement with each other throughout the calibration range.

The accuracy and precision of the method were evaluated by analyzing QC samples at four different concentrations of (phospho)peptides in three replicates. The results are listed in Table S1 (see ESM). The intra- and inter-day precisions and accuracy were satisfactory for all the QC samples [43]. The evaluation of three-cycle freeze-thaw, 48 h post-preparative

Fig. 5 Time-dependent stoichiometry of ERK1/2 phosphorylation and Bcl-2 expression level in MCF-7/WT cells with the incubation of DOX a and the effect of inhibitor U0126 on ERK1/2 and Bcl-2 in MCF-7/WT cells treated with DOX for 30 min and 48 h, respectively. b Significant differences compared with control are indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. c Protein levels in MCF-7/ADR cells using the same procedure

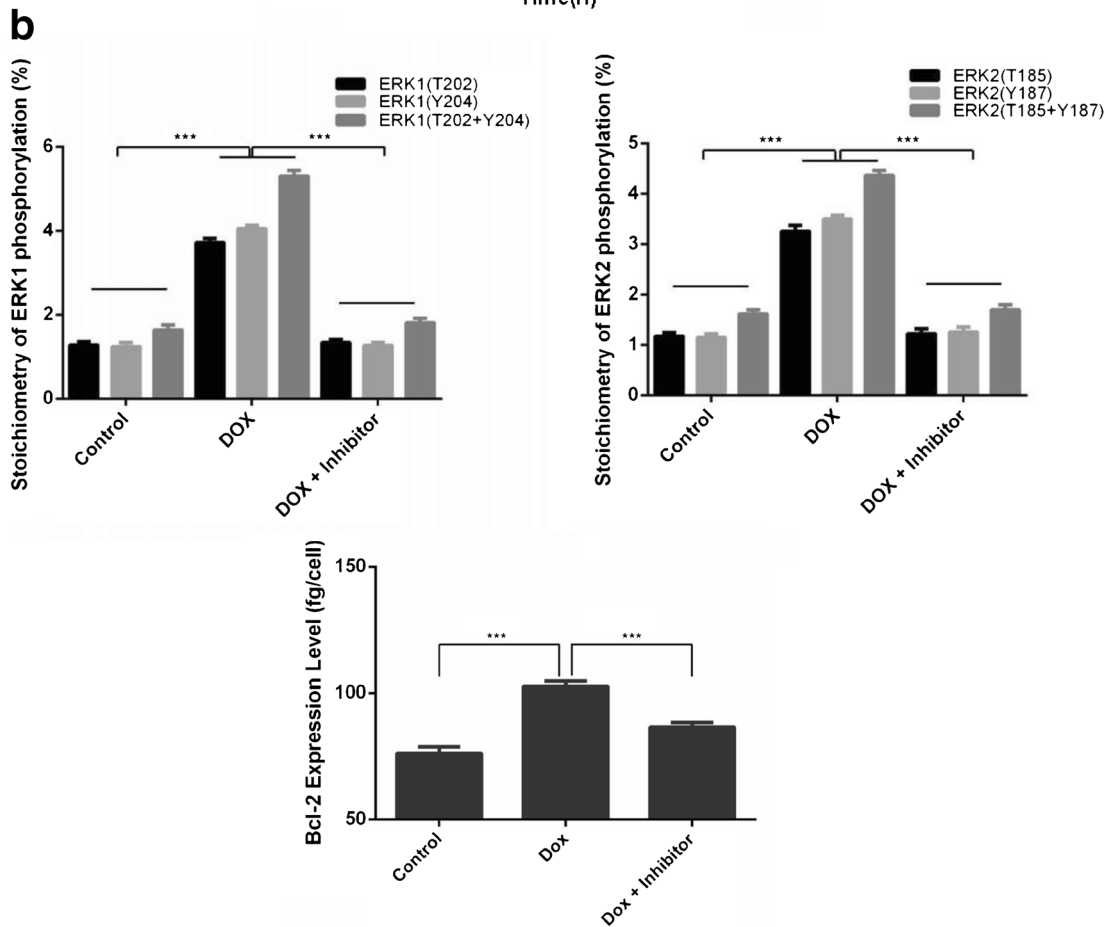
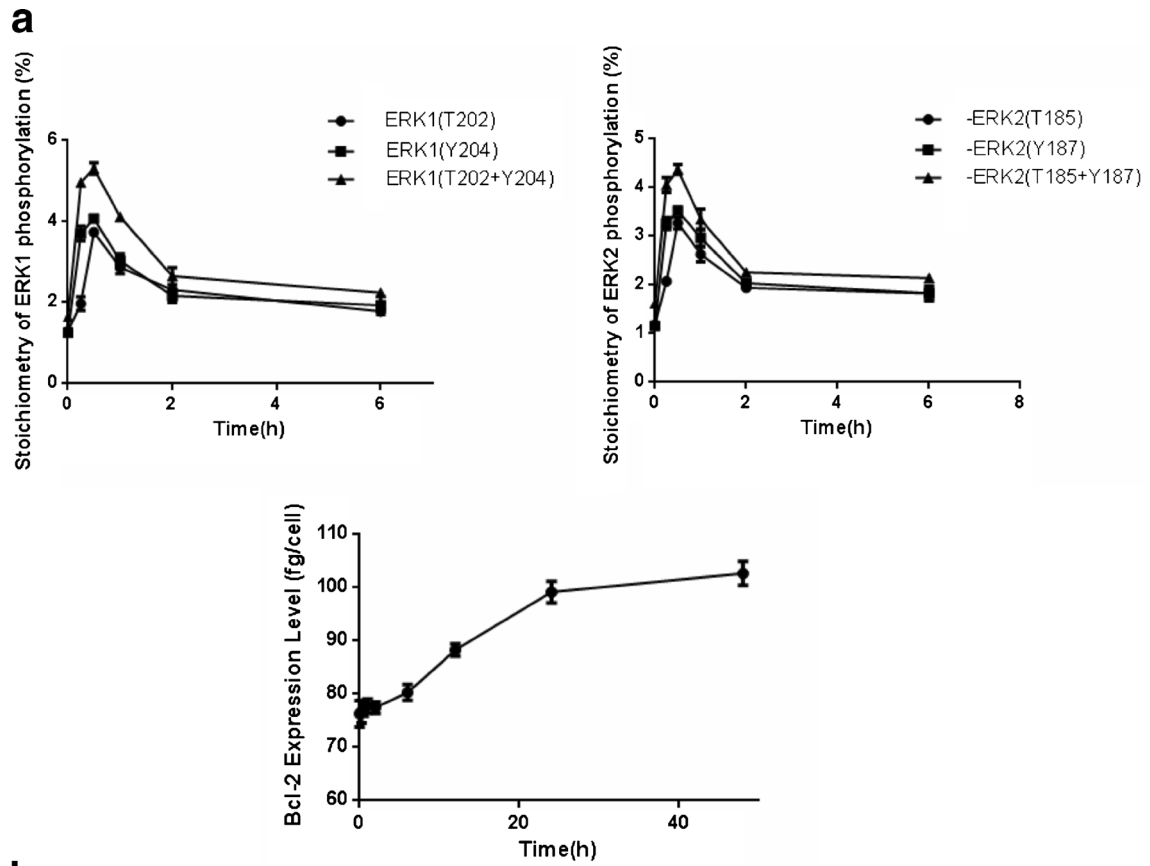
(4 °C) and 12 h room temperature stabilities, was also performed here. The outcome demonstrated acceptable stability of (phospho)peptides (data not shown).

Quantitative analysis of ERK1/2 and Bcl-2 in breast cancer cells and tissue samples

Using the LC-MS/MS-based targeted proteomics assay, the protein levels of ERK1/2 and Bcl-2 and the site-specific phosphorylation stoichiometry of ERK1/2 were accurately quantified in MCF-7/WT and MCF-7/ADR cells (Table 2). Notably, isobaric phosphopeptides were determined using their common product ions by chromatographic separation. The result was subsequently confirmed using their site-specific product ions (ESM Table S2). As shown in the tables, MCF-7/ADR cells had a slightly increased ERK1/2 level compared to their parental cells, whereas the extent of ERK1/2 phosphorylation in MCF-7/ADR cells was much greater (i.e., ~3-fold). This phenomenon suggested that the active, but not the total level of ERK1/2, may contribute to drug resistance.

In addition, the phosphorylation stoichiometry of both ERK1 and ERK2 increased in MCF-7/ADR cells. The unequal enhancement provided a compelling evidence for the distinct role of these two kinases in biological system. As described previously, ERK1 and ERK2 have similar amino acid composition and are often not distinguished [46]. Apparent difference in their relative not absolute abundance has only been reported in a few studies. ERK2 appeared to be the mediator of the proliferative signal, whereas ERK1 had an antagonistic function to ERK2 [10]. Thus, differential ERK1 and ERK2 phosphorylation can have a profound effect on the readout from the ERK1/2 pathway, resulting in distinct cellular outcomes. Determination of the contribution of each ERK to the cellular response will help us to reassess their roles in various biological processes. In this study, the finding of a greater enhancement of ERK1 phosphorylation in MCF-7/ADR cells suggested that its antagonistic function may be more induced in the drug resistance acquisition.

Furthermore, the levels of differential phosphorylated forms of ERK1/2 were also different. Taken into account the fact that partially phosphorylated forms may be active as well as di-phosphorylated form [47], the presence of mono-phosphorylated ERK1/2 and their differential



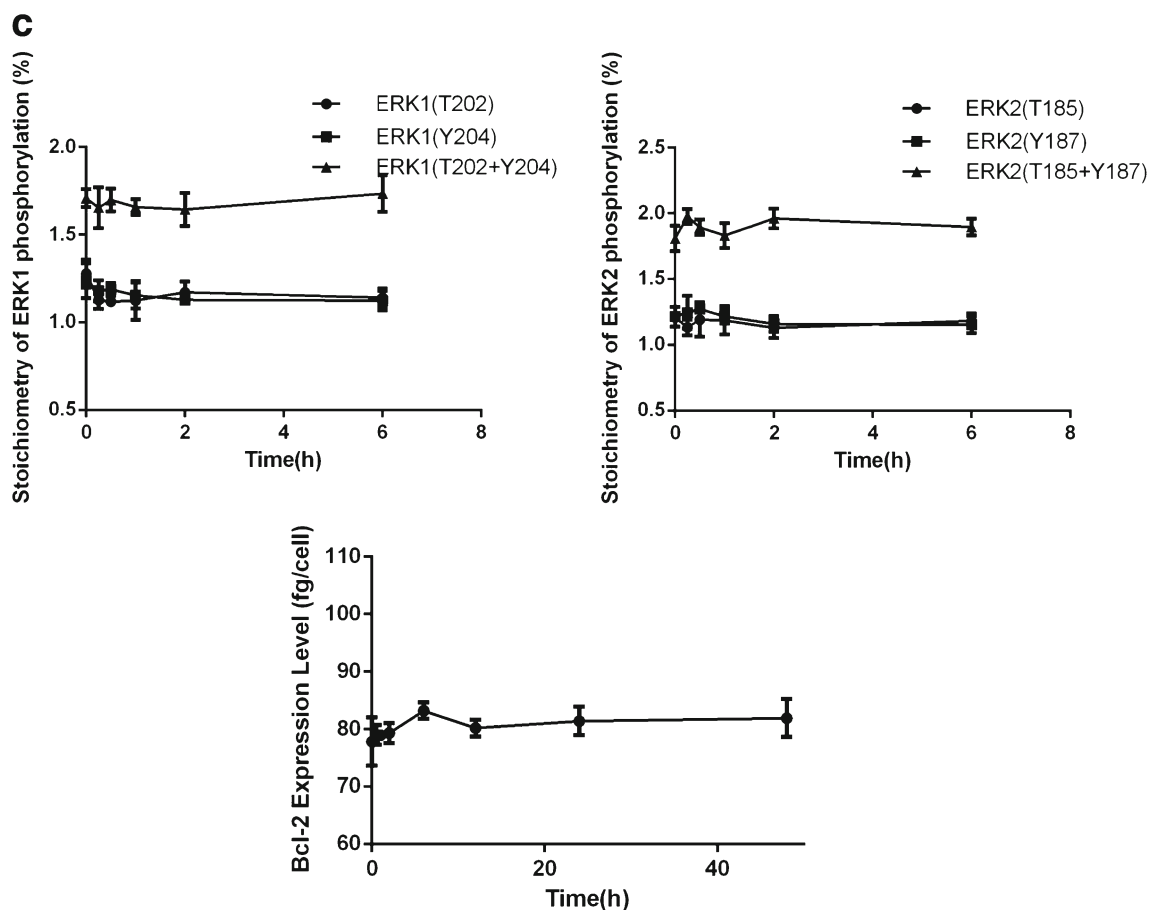


Fig. 5 (continued.)

abundance suggested that these phosphorylated forms may affect biological functions through the mechanisms that were dependent on the phosphorylation site. Indeed, the association between tyrosine-phosphorylated form of ERK and drug resistance has been reported [13]. Time-course analysis also provided site-specific phosphorylation profiles, which will be described in the next section. Future studies need to address how the balance between these phosphorylated forms affected the signaling dynamics of pathway.

The ERK1/2 result when combined with the data suggesting an overexpression of Bcl-2 in MCF-7/ADR cells (Table 2) supported the previous observations of ERK1/2 involvement in Bcl-2-mediated anti-apoptosis and MDR acquisition using antibody-based techniques [48–50]. Correspondingly, the dose-log response curves in previous studies reported the half maximal inhibitory concentration (IC_{50}) of DOX in MCF-7/WT and MCF-7/ADR cells as 569.7 ± 30.4 nM and 35.6 ± 2.4 μ M [3]. The resistance index (RI) was 62.5 in MCF-7/ADR cells relative to the parental MCF-7/WT cells [46].

We also performed conventional analytical assays including fluorescence microscopy and Western blotting in the

present study (Fig. 3). Compared with their qualitative/semi-quantitative results, making it difficult to discern changes in phosphorylated protein expression [41], targeted proteomics approach can determine protein levels and phosphorylation extent in values. In addition, both of antibody-based methods cannot easily discern the protein isoforms and their different phosphorylated forms. They usually target only one analyte at a time because method development process of multiple colors is complicated due to fluorescence spectral overlap [51]. Furthermore, one could compare the expression levels of each isoform only when the antibody recognizes an identical epitope per isoform [52]. The success of LC-MS/MS-based targeted proteomics in the quantification of phosphopeptides containing more than one phosphorylation site, especially isobaric phosphopeptides [16, 17], and the determination of the extent to which phosphorylation status has changed confirmed this technique as an alternative tool to resolve such post-translational modification issues.

Using the LC-MS/MS-based targeted proteomics assay, breast tissue samples were also subjected to analysis. As shown in Fig. 4, patients with suspected drug resistance have significantly greater levels of ERK1/2 phosphorylation compared to those counterparts. The distribution profile of

individual ERK1/2 phosphorylated forms is consistent with the previous observation in cells. This result demonstrated that the quantitative information of ERK1/2 and their phosphorylated forms including isobaric ones may lead to better cancer patient stratification with diagnostic significance. However, additional, larger patient cohorts must be tested to determine whether ERK1/2 and their differential phosphorylated forms can be used alone as independent factors or in combination with other markers and whether they are more selective for specific clinical applications in breast cancer diagnosis and treatment. In addition, the association between ERK1/2 expression levels and disease progression implied that ERK1/2 could be an attractive therapeutic target.

Quantitative time-course analysis of ERK/Bcl-2-mediated anti-apoptosis

In this study, the quantitative feature of LC-MS/MS-based targeted proteomics was further implemented to closely monitor ERK1/2 and Bcl-2 in the development of drug resistance. MCF-7/WT cells were incubated with DOX and then explored for the amounts of ERK1/2 and Bcl-2. As a result, there was no significant change of ERK1/2 protein levels after the treatment of DOX. The phosphorylation of ERK1/2 was rapidly stimulated and reached a peak within the first 30 min, whereas the level of Bcl-2 gradually increased in a time-dependent manner (Fig. 5a). This phenomenon was consistent with the DOX-induced ERK1/2 activation and Bcl-2 overexpression as previously reported [2, 53]. In addition, it is interesting to observe that threonine phosphorylation lagged behind tyrosine phosphorylation, suggesting a dual phosphorylation reaction process [54]. In comparison, the treatment of DOX had less effect on ERK1/2 phosphorylation in MCF-7/ADR cells and there was no evidence of an enhancement in Bcl-2 expression. To further confirm these results, ERK1/2 phosphorylation was inhibited using an inhibitor U0126 (Selleck Chemicals, Houston, TX, USA). As shown in Fig. 5b, DOX-induced ERK1/2 activation was significantly reduced in MCF-7/WT cells pretreated with U0126, whereas Bcl-2 increase was only partially inhibited. These findings further provided evidence to that ERK/Bcl-2-mediated anti-apoptosis pathway participated in the drug resistance acquisition. The corresponding Western blotting was also performed for a comparison (see ESM Fig. S2). As a control, MCF-7/ADR cells were further studied using the same procedures. No significant change of ERK and Bcl-2 was observed (Fig. 5c).

Conclusions

In this study, ERK/Bcl-2-mediated anti-apoptosis was investigated in both breast cancer cells and breast tissue samples using a LC-MS/MS-based targeted proteomics assay. In particular, ERK

isoforms and their differential phosphorylated forms can be distinguished and detected simultaneously. In agreement with the previous results regarding ERK/Bcl-2-mediated anti-apoptosis and MDR development, the change of protein and phosphorylation levels was observed. Furthermore, this change was monitored over time. By comparison with conventional analytical methods, the quantitative and targeted nature of targeted proteomics approach was further illustrated in the study of cellular pathway. Finally, the feature of targeted proteomics to investigate biological events needs further elucidation by the involvement of more protein components.

Acknowledgments The authors wish to gratefully acknowledge the National Natural Science Fund (21175071), the project sponsored by SRF for ROCS, SEM (39) and the Jiangsu Six-Type Top Talents Program (D), and the Open Foundation of Nanjing University (SKLACLS1102) awarded to Dr. Chen and the Fundamental Research Funds for the Central Universities (021414380231) to Ms. Yang.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Breast tissue collection in this study was approved by the Institutional Review Board of Nanjing Medical University. Informed consent was obtained from all individual participants included in the study.

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