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Comparison of LC-MS/MS-based targeted proteomics and conventional analytical methods for monitoring breast cancer resistance protein expression

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ABSTRACT

Aims: Multidrug resistance is a major obstacle in chemotherapy, which is mainly caused by the overexpression of ATP-binding cassette (ABC) transporters. Breast cancer resistance protein (BCRP) is one of the ABC transporters and is strongly associated with multidrug resistance. Results of studies on BCRP and multidrug resistance are always uncomparable and contradictory, which may be stem from the disadvantages of qualitative and semi-quantitative techniques. In addition, there are few literatures studying at low resistance level which is more similar to the clinical situation. Thus, it is imperative to develop a quantitative method to quantitate the expression of BCRP accurately and reveal its relationship with multidrug resistance.

Methods: SMMC-7721, MCF-7 and HepG-2 were induced by different concentrations of mitoxantrone, doxorubicin and methotrexate respectively to establish resistance cells. An advanced liquid chromatography linked to tandem mass spectrometry (LC-MS/MS) based method with surrogate peptide was developed and validated for determining BCRP at low resistant cells. The amount of BCRP was also evaluated by real-time-polymerase chain reaction (RT-PCR) and Western Blot (WB).

Key findings: The LC-MS/MS-based method we developed is more sensitive and stable than the similar methods and can monitor the slight variation of BCRP expression accurately and sensitively, while RT-PCR and WB cannot.

Significance: This study provides a solid foundation for understanding the development of drug resistance in cells and can be used to explain the conflicting results of published studies. Moreover, clinical multidrug resistances are mostly at low levels, which have not been discussed in current quantitative studies of BCRP.

1. Introduction

The development of multidrug resistance (MDR) is the main reason for the failure of chemotherapy. The active efflux of drugs by ATP-binding cassette (ABC) drug transporters is an important factor of MDR [1–3], which decreases the accumulation of intracellular drugs and reduces the therapeutic effect. Currently, breast cancer resistance protein (BCRP, one of the ABC transporters) has been drawing more and more attention, whose overexpression has been confirmed as the main reason for the resistance of numerous drugs, such as mitoxantrone (MX), roxithromycin, acetabular ethylglycoside and topotecan [4,5]. In order to achieve individualized breast cancer chemotherapy, BCRP expression is used as a crucial reference index to predict the effect of drugs. Meanwhile, BCRP is strongly associated with the chemotherapy sensitivity of various tumors, such as ovarian cancer, acute myeloid leukemia and non-small cell lung cancer [6–8].

At present, BCRP detection methods mainly include real-time-polymerase chain reaction (RT-PCR), evaluating protein from the transcription level of mRNA, and Western Blot (WB), semi-quantitating protein based on the principle of immunoreactions. The non-unified criterions are more likely to lead to uncomparable or even opposite results among different laboratories. For example, Ross et al. indicated [9] that cells transfected with BCRP were methotrexate (MTX) resistant and BCRP was highly overexpressed in MCF7/MX cells, but Volk et al. [10] suggested that BCRP was neither involved in MTX cross-resistance cells nor overexpressed in the MCF7/MX cells. The contradiction may be caused by the short of specificity and repeatability of WB and RT-PCR [11,23].

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Additionally, MDR is predominantly studied at the high resistance levels in the existing literatures which is inconsistent with the clinical situation, thus it is recommended to study the relationship between BCRP expression and drug resistance at low resistance index (RI, the ratio of half maximal inhibitory concentration before and after drug resistance) levels [12,30]. However, at low resistance levels, the amount of BCRP changes slightly, which is not sensitive enough to be accurately monitored by RT-PCR and WB. Therefore, it is imperative to develop a more accurate and sensitive BCRP quantification method.

Quantitative targeted absolute proteomics (QTAP) is a technology established on biological mass spectrometry, which has an outstanding ability to isolate and detect target proteins. The method involves the enzymatic hydrolysis of the samples and the substitution of the target protein with a surrogate peptide in detection. Then the stable isotope labeled peptide is added as the internal standard (IS) to achieve quantitative determination of the surrogate peptide and to calculate the amount of corresponding target protein. Multiple reaction monitoring [13] (MRM) filters fragment and precursor ions used for surrogate peptide quantification. Hence, QTAP is able to detect specific peptide from biological samples with target protein and has been successfully applied to biomacromolecule quantification (such as protein and nucleic acid).

In our study, a more accurate and stable LC-MS/MS-based method for the quantification of BCRP with surrogate peptide (SSLLDVLAAR) was developed and validated. Then, different concentrations of adriamycin (ADM), MX and methotrexate (MTX) were utilized to induce low drug resistance levels of SMMC-7721, HepG-2 and MCF-7 cells. Based on the LC-MS/MS method, the relationship between BCRP expression and drug resistance in tumor cells was studied and the results were compared to those obtained by RT-PCR and WB.

2. Materials and methods

2.1. Chemicals and reagents

Adriamycin (ADM, CAS: 25316-40-9) was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). Methotrexate (MTX, CAS: 59-05-2) and mitoxantrone (MX, CAS: 70476-82-3) were purchased from National Institutes for Food and Drug Control (Beijing, China). The synthetic peptide SSLLDVLAAR (≧99.5% purity) and the stable isotope-labeled internal standard SSLLDVL (13C,15) NAAR (≧99.5% purity) were purchased from Jill Biochemical Co., Ltd. (Shanghai, China).

Sequencing grade modified trypsin was purchased from Merck (Madison, USA). The protein quantification bicinchoninic acid (BCA) kit was purchased from Pierce Biotechnology (Rockford, USA). Ammonium bicarbonate (NH_4HCO_3) was purchased from Qiangshun Chemical Reagent Co., Ltd. (Shanghai, China). Methanol and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Human serum albumin (HSA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) was purchased from Amersco (Washington State, USA). Trifluoroacetic acid (TFA), Tris-HCl, iodoacetamide (IAA) and Triton X-114 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was purified and deionized by Milli-Q system purchased from Millipore (Bedford, MA, USA). Phosphate buffered saline (PBS) was purchased from the Beyotime Institute of Biotechnology (Jiangsu, China). SMMC-7721, HepG-2 and MCF-7 cells were purchased from Cell Bank of Chinese Academy of Sciences (Beijing, China). Trizol was purchased from Gibco BRL, Life Technologies, Inc., Rockville (MD, USA). RPMI-1640, trypsin containing 0.25% EDTA and Dulbecco's modified eagle media (DMEM) were purchased from Gibco (California, USA). Fetal bovine serum (FBS) was purchased from Invitrogen (Burlington, Canada).

2.2. Identification of surrogate peptide

Surrogate peptide for BCRP was selected via *in-silico* process and experimental verification. Briefly, an intact protein sequence was gained from NCBI and then a set of potential surrogate peptides were produced by Skyline. After the verification of peptide's specificity by UniProt and Blast, surrogate peptides were obtained preliminarily.

For further verifying the specificity of surrogate peptides, the blank matrix of HSA was configured in the standard solution preparation procedure and then was analyzed by mass spectrometry.

An appropriate surrogate peptide was validated by a triple quadrupole mass spectrometer and was utilized for BCRP quantification. In order to establish optimum MRM, the appropriate mass shift of surrogate peptide was determined and optimized by artificial injection of isotope-labeled synthetic peptide. For surrogate peptide, the highest intensity mass conversion was chosen. In this condition, the response, sensitivity and selectivity of this quantitative method were all the best.

2.3. LC–MS/MS method development and validation

Standard stock solution of surrogate peptide (2 mg/mL) was prepared by dissolving the peptide in deionized water. Then the standard stock solution was reserved in different brown glass tubes and was stored at −20 °C. The isotope-labeled peptide, whose preparation procedure was the same with standard stock solution, was used as an internal standard (IS) and was then diluted by the mobile phase (water: acetonitrile = 50:50, v/v) until its concentration was 30 ng/mL.

For the preparation of calibration curve, digested HSA was used as blank matrix. The standard solution was prepared by serial gradient dilution of the stock solution to obtain the following concentrations: 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, 50, and 100 ng/mL for calibration values. Then a double blank without any peptide and a zero-standard containing no analytic peptide but IS, were included in the calibration curve. Quality control (QC) samples, lower limit of quantification (0.1 ng/mL, LLOQ), low QC (0.16 ng/mL, LQC), mid-QC (4 ng/mL, MQC) and high QC (80 ng/mL, HQC), were mixed with the SIL-IS.

2.4. Resistance cell lines culture and protein extraction

SMMC-7721 was cultured within RPMI-1640 media. HepG-2 and MCF-7 were cultured within DMEM media. All the cells were supplemented by 10% FBS, 100 units penicillin and 100 μg/mL of streptomycin at 37 °C and 5% CO_2. The MTT method (one of the cell viability tests) was used to measure the half-inhibitory concentration (IC_{50}) of MTX, ADM and MX on each cell line. The average of IC_{50} was used as the beginning induction concentration for each drug: MTX is 0.319 μg/mL, MX is 0.209 μg/mL, ADM is 0.168 μg/mL. Then SMMC-7721, HepG-2 and MCF-7 cells were treated with drugs of gradient concentrations gradually to develop resistance cells (MCF-7/MX, HepG-2/MX, SMMC-7721/MX, MCF-7/MTX, HepG-2/MTX, SMMC-7721/MTX, HepG-2/ADM, MCF-7/ADM and SMMC-7721/ADM), until the cells were able to grow at 1, 2, and 3 times of IC_{50} steadily.

The cells were evenly divided into three parts for RT-PCR, WB and LC-MS/MS analysis respectively, and the cell numbers were counted by Trypan Blue staining. Firstly, the cells were centrifuged at 1500 × *g* for 10 min and were re-suspended in 500 μL of 1% Triton X-114 extraction buffer with protease inhibitor cocktail (2 mM EDTA in 50 mM Tris-HCl, 1 mM DTT, pH 7.4). The sample was incubated on ice for 30 min, shaken every 10 min for 1–2 min, and incubated at 37 °C for 10 min. Afterwards, samples were centrifuged at 10,000 × *g* for 10 min to separate the detergent and aqueous phases. To achieve a complete extraction, 500 μL of 1% Triton X-114 extraction buffer was added to the aqueous phase and 500 μL of 0.06% Triton X-114 wash buffer was added into the detergent phase. The incubation and centrifugation steps were repeated again. The supernatant was removed and the retained protein sample was condensed in cold acetone at −20 °C for 1 h. Finally, the protein sample was re-suspended and was stored at −80 °C before analyzing. BCA Protein Assay Kit was applied to determine total membrane protein concentration in the isolated membrane fraction.

2.5. Trypsin digestion

Extraction of membrane protein was diluted to 2 mg/mL, of which 100 μL was mixed with 50 μL of 50 mM NH_4HCO_3 buffer. The mixture was eddied for 2 min and subsequently reduced with 10 mM DTT, incubated at 60 °C for 20 min. After alkylated with 50 mM IAA in 50 mM ammonium, the outcome was then incubated at room temperature for 6 h in the dark. Sequencing grade modified trypsin was added into the tube according to the ratio of protein: trypsin (20:1, w/w), and the tryptic digestion was carried out at 37 °C for 24 h in the dark. Digestion was terminated by 20 μL of 10% TFA. The tryptic digest was centrifuged at 16,000 × *g*, 4 °C for 15 min. Then the supernatant was transferred to a clean tube. After evaporated to dryness at 45 °C under a mild stream of nitrogen, the residue was redissolved by 100 μL of mobile phase and was centrifuged at 16,000 × *g*, 4 °C for 15 min. Afterwards, 5 μL of the supernatant was analyzed by the LC-MS/MS system.

2.6. BCRP quantitation by LC-MS/MS

A Waters ACQUITY UPLC system which was composed of a triple quadrupole mass spectrometer and equipped with a sample management system, and a programmable column management system was used for sample analysis. The chromatographic conditions of BCRP were consistent. The liquid chromatography separations were performed on a BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters, USA) at 35 °C. The mobile phase consisted of solvent A (water: 0.1% formic acid, v/v) and solvent B (methanol: 0.1% formic acid, v/v). A linear gradient with a flow rate of 0.3 mL/min was applied in the following way: 90% A (0–1 min), 90%–10% A (1–3 min), 10% A (3–4 min) and 90% A (4–5 min). The inject volume was 5 μL.

The mass spectrometer was equipped with the electrospray ionization (ESI) and was operated in the positive ion mode to monitor the *m/z* transitions for the surrogate peptide and IS. Gas temperature was 350 °C and spray voltage was 3 kV. The monitoring ion of BCRP surrogate peptide was 522.8 → 644.4. IS monitoring ion was 526.3 → 651.1. The crushing voltage was 20 V and the collision energy was 20 eV.

Data was processed via the analyst 1.8.2 software (Waters UNIFI, Waters, Milford, MA) by integrating the appropriate peak areas which were generated from the reconstructed ion chromatogram for the surrogate peptide and the SIL-IS. The amount of surrogate peptides was quantified by calculating the ratio of the peak area to the SIL-IS peak area.

2.7. Measurement of BCRP mRNA by RT-PCR

Total RNA and its resistant subclones of SMMC-7721, HepG-2 and MCF-7 cells were extracted by triazole. Reverse transcription was performed according to the PrimeScript RT reagent suite protocol. RT-PCR was performed based on the SYBR premix kit protocol using iQ5 RT-PCR and ABI 7500 instrument. The following primers were used for BCRP: ABCG2 (F), 5′-TGGCTGTCATGGCTTCAGTA-3′, ABCG2 (R), 5′-GCCACGTGATTCTTCCACAA-3′, GAPDH (F), 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, GAPDH (R), 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. GAPDH was used as a reference for correction.

2.8. Measurement of BCRP by Western Blot

The total protein was extracted by a conventional method and the protein concentration was measured by Coomassie Brilliant Blue method. 50 μg of protein was processed as follows: denatured, electrophoresed, transferred to nitrocellulose membrane, stained with ponceau red, sealed with 5% skim milk, washed with I anti-BCRP (1:1000) or GAPDH (1:5000) at 4 °C overnight, and incubated with root peroxidase HRP-labeled II anti-IgG (1:4000) for 2 h at room temperature. Subsequently, the ELC chemiluminescence kit was auto-developed in a dark room. The experiment used GAPDH as a reference and the BandScan 5.0 software was used to measure the gray value of the specific band. The ratio of the gray value of the target band to the gray value of the corresponding GAPDH indicated the amount of BCRP.

```
1   MSSSNVEVFI  PVSQGNTNGF  PATASNDLKA  FTEGAVLSFH  NICYRVKLKS  GFLPCRKPVE
61  KEILSNINGI  MKPGLNAILG  PTGGGKSSLL  DVLAARKDPS  GLSGDVLING  APRPANFKCN
121 SGYVVQDDVV  MGTLTVRENL  QFSAALRLAT  TMTNHEKNER  INRVIQELGL  DKVADSKVGT
181 QFIRGVSGGE  RKRTSIGMEL  ITDPSILFLD  EPTTGLDSST  ANAVLLLLKR  MSKQGRTIIF
241 SIHQPRYSIF  KLFDSLTLLA  SGRLMFHGPA  QEALGYFESA  GYHCEAYNNP  ADFFLDIING
301 DSTAVALNRE  EDFKATEIIE  PSKQDKPLIE  KLAEIYVNSS  FYKETKAELH  QLSGGEKKKK
361 ITVFKEISYT  TSFCHQLRWV  SKRSFKNLLG  NPQASIAQII  VTVVLGLVIG  AIYFGLKNDS
421 TGIQNRAGVL  FFLTTNQCFS  SVSAVELFVV  EKKLFIHEYI  SGYYRVSSYF  LGKLLSDLLP
481 MTMLPSIIFT  CIVYFMLGLK  PKADAFFVMM  FTLMMVAYSA  SSMALAIAAG  QSVVSVATLL
541 MTICFVFMMI  FSGLLVNLTT  IASWLSWLQY  FSIPRYGFTA  LQHNEFLGQN  FCPGLNATGN
601 NPCNYATCTG  EEYLVKQGID  LSPWGLWKNH  VALACMIVIF  LTIAYLKLLF  LKKYS
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Fig. 1. The protein sequence of BCRP obtained from NCBI.

2.9. Statistical analysis

Social Sciences SPSS for Windows version 22.0 was used for the statistical analysis. Analysis of Variance (ANOVA) was used to evaluate the differences between groups and the significance level *P* value was set to 0.05.

3. Results and discussion

3.1. Selection of the surrogate peptides

The selection of surrogate peptide was the first and the most critical step in QTAP, which was firstly processed by software tools and then verified by experiments.

In the *in-silico* process, the full human BCRP sequence (Fig. 1) could be obtained from NCBI, combined with Skyline (predict the polypeptide of target protein and the mass spectrometry information of each peptide), UniProt (exclude uncertain and unstable sites), PeptideAtas (predict the MRM of a specific peptide), BLAST (verify the specificity of peptide theoretically) and other databases, surrogate peptide could be obtained preliminarily. Ulteriorly, the peptide was ensured to meet these requirements: (1) To ensure the mass range of peptide was detectable by triple quadrupole MS, only peptide with 7–22 amino acids was qualified. (2) Continuous sequence of arginine (R) or lysine (K), such as: RR, KK, RK, or KR, should be excluded to avoid incomplete trypsin digestion. (3) No transmembrane region; no post-translational modification areas; no single nucleotide polymorphism or mutation site; no easily oxidized amino acid residue. (4) For fear of losing peptides in operation, the best proportion of hydrophobic amino acids was 25%–50% to make sure that the peptide could be dissolved in water completely. The candidate peptides screened by the process were SSLLDVLAAR and ENLQFSAALR.

Purpose of experimental verification was to ensure that the surrogate peptide had the highest intensity and there was no interference signal from the biological matrix. In this study, 5% HSA in PBS (blank matrix) without surrogate peptide or its SIL-IS was digested and analyzed by the LC-MS/MS system, the chromatogram was shown in Fig. 2a (double blank) and Fig. 2b (no surrogate peptide but SIL-IS). Then, a proper working solution without any diluted surrogate peptide was prepared, and the surrogate peptide and its SIL-IS was added to it before tryptic digestion, the chromatogram was shown in Fig. 2c and Fig. 2d. According to the above experimental verification, SSLLDVLAAR was identified as the surrogate peptide because of the higher intensity and no interference, which was in accordance with the studies published previously [14–16].

3.2. Blank matrix selection

Considering the possible interference of the sample matrix in the

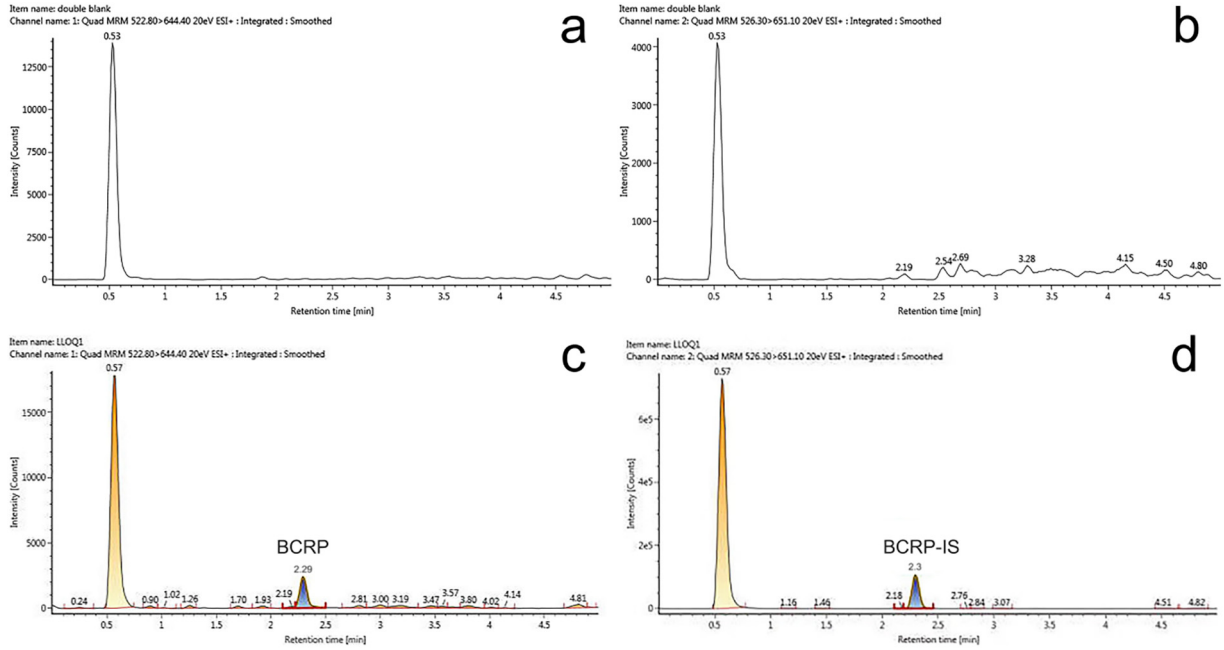

Fig. 2. HSA had no interference in the ion channel of surrogate peptide and the IS.

measurement, it was preferred to use the samples without target protein as blank matrix [17]. However, it was difficult to ensure that the target protein was completely removed and no new impurities were included. Since HSA was a commonly used matrix and has been reported in plenty of studies [18,19], it was applied in our study. Experiments were carried out to verify the matrix effect as well.

The result showed that the digested 5% HSA in PBS would not interfere the quantitative analysis of the surrogate peptide and the IS by comparing the chromatogram of the digested 5% HSA in PBS, surrogate peptide (Fig. 2c) and its IS (Fig. 2d). Furthermore, the surrogate peptide selected in the experiment was not disturbed by HSA or the proteins in the membrane protein samples during the measurement process. Finally, the digested 5% HSA in PBS was selected and further verified as the blank matrix.

3.3. LC–MS/MS analysis and method validation

There was a positive linear correlation between peptide concentration and the analytical signal for the entire validation range (0.096–96 fM for BCRP). The correlation coefficient (R^2) for the calibration curve ranged between 0.9982 and 0.9995 (weight: $1/X^2$). The LLOQ of our analytical assay for BCRP was 0.096 fM, which was lower than the LLOQ values from previously published LC-MS/MS method [15–21].

The precision and accuracy of the assay were assessed by observing the response of the QC samples with four different concentrations of surrogate peptide (n = 15). The precision was reflected by the percent coefficient of variation (%RSD). The accuracy was obtained by comparing the average calculated concentrations to their nominal values (% RE). Intra-day accuracy and inter-day accuracy for peptide in validation matrix were within the required range of ± 15% (± 20% at LLOQ), and intra-day precision and inter-day precision were within the required range of ± 15%. Details are listed in Table 1. Stability was evaluated by analyzing samples exposed to different conditions and accuracy for peptide was within the required range of ± 15%, details are listed in Table 2. The stability and precision of samples were evaluated in this study as well. The results of this study showed that the inter-assay precision (n = 3) and intra-assay (n = 5) precision were within ± 15% (Table 3, HepG-2/MTX). As shown in Table 4 (HepG-2/MTX), the precisions of stability were within ± 15%, which indicated that the samples were stable under such conditions and the LC-MS/MS method for the quantification of BCRP amount in samples was reliable.

Triton X-114, which was usually used for the extraction of membrane proteins from cells was used to extract BCRP from the cells [29]. Furthermore, the extraction procedure was repeated three times to make sure the complete protein extraction [18].

3.4. Digestion efficiency

Experiments were designed to explore optimal condition for the digestion. The enzymatic hydrolysis temperature was 37 °C and the enzymatic hydrolysis time of the membrane protein was 2 h, 4 h, 16 h and 24 h, respectively. The efficiency of 24 h was assumed to be 100% at first since the tryptic digestion time of ABC transporters in most studies were 24 h [22,23]. Then the efficiency of other time was compared with 24 h. It was observed that the enzymatic hydrolysis times of different cell types were diverse from each other. For SMMC-7721, the efficiency gradually increased from 2 h to 32 h, while there was a fluctuation for HepG-2, as shown in Fig. 3. In all, digestion was carried out in the dark at 37 °C for 24 h to make sure that the target protein in the sample was completely hydrolyzed by the enzyme.

3.5. Comparison of quantitative results between LC-MS/MS analysis and conventional analytical methods

The results of BCRP quantitation by LC-MS/MS are shown in Fig. 4(a, b, c). Statistically significant differences ($P < 0.05$) between parental cell lines and drug resistant cell lines were observed. For example, the expression of BCRP increased by 2.0 times ($P < 0.05$) in HepG-2 under the induction of MTX ($3 \times IC_{50}$), and increased by 3.0 times ($P < 0.05$) in SMMC-7721 under the induction of MTX ($2 \times IC_{50}$).

It could be found that RT-PCR could not monitor the slight variation of BCRP at low RI. As shown in Fig. 4(d, e, f), no statistically significant variation of BCRP mRNA was observed with the development of drug resistance at a later stage, which was obviously different from the results of LC-MS/MS. In SMMC-7721/MTX cell lines, BCRP expression increased with the accumulation of drug concentration ($P < 0.05$), but it was not observed by RT-PCR and the level of BCRP mRNA decreased in SMMC-7721/ADM ($2 \times IC_{50}$) abnormally. The results of these two

Table 1
Accuracy and intra-day, inter-day precision.

QC level	LLOQ			LQC			MQC			HQC		
Theoretical concentration	0.1 ng/mL			0.16 ng/mL			4 ng/mL			80 ng/mL		
Intra-day precision												
Batch	1	2	3	1	2	3	1	2	3	1	2	3
Mean	0.998	0.101	0.995	0.162	0.168	0.158	3.89	3.89	3.87	81.4	78.9	80.8
Accuracy (%)	99.8	101	99.5	101	105	98.7	97.3	97.3	96.9	101	98.7	101
RSD (%)	5.30	7.10	3.80	8.50	6.20	7.71	2.04	1.80	0.65	1.70	2.27	0.90
Inter-day precision												
Mean	0.997			0.157			3.99			79.9		
Accuracy (%)	99.7			98.1			99.8			99.9		
RSD (%)	8.10			6.45			1.73			2.80		

Table 2
Stability of samples under different conditions.

QC levels	Stability tests	Conditions			
		6 h at room temperature before processing	3 freeze-thaw cycles (−20 °C)	48 h at room temperature after processing	24 h at auto-sample condition after processing
LQC (0.16 ng/mL)	Mean	0.169	0.166	0.149	0.147
	Accuracy (%)	105	103	93.1	91.9
MQC (4 ng/mL)	Mean	3.88	3.91	3.69	3.39
	Accuracy (%)	96.9	97.8	92.3	84.8
HQC (80 ng/mL)	Mean	82.1	83.5	74.6	72.3
	Accuracy (%)	103	104	93.2	90.4

Table 3
Intra-day, inter-day precision of the real samples (HepG-2/MTX).

Cell lines	Parental cell line			IC_{50}			2 × IC_{50}			3 × IC_{50}		
Intra-day precision												
Batch	1	2	3	1	2	3	1	2	3	1	2	3
Mean (fmol/mg)	0.0998	0.0986	0.0992	0.103	0.100	0.105	0.185	0.186	0.182	0.384	0.372	0.391
RSD (%)	2.60	7.40	6.81	7.35	5.52	3.97	2.93	4.64	8.10	6.65	7.44	5.41
Inter-day precision												
Mean (fmol/mg)	0.099			0.101			0.181			0.380		
RSD (%)	8.71			5.72			1.65			8.05		

Table 4
Stability of the real samples (HepG-2/MTX) under different conditions.

Cell lines	Stability tests	Conditions			
		6 h at room temperature before processing	3 freeze-thaw cycles (−20 °C)	48 h at room temperature after processing	24 h at auto-sample condition after processing
Parental cell line	Mean (fmol/mg)	0.0982	0.0987	0.0988	0.0990
	RSD (%)	5.11	7.44	6.27	3.39
IC_{50}	Mean (fmol/mg)	0.102	0.101	0.101	0.100
	RSD (%)	2.95	3.35	5.62	4.60
2 × IC_{50}	Mean (fmol/mg)	0.184	0.182	0.180	0.181
	RSD (%)	3.70	6.67	4.18	3.97
3 × IC_{50}	Mean (fmol/mg)	0.382	0.379	0.380	0.378
	RSD (%)	8.10	5.33	6.75	8.61

methods were markedly different mainly because that the level of mRNA was not always consistent with the protein expression. Meanwhile, the amplification in RT-PCR also raised the possibility of error. Prasad et al. [24] even indicated that the level of BCRP was not associated with mRNA expression. Therefore, the results of RT-PCR could not reflect BCRP expression accurately.

The results of WB also showed that BCRP expression did not change significantly in drug resistant cell lines and the result of BCRP expression in MCF-7 was shown in Fig. 5. For example, no obvious difference could be observed in HepG-2/MTX, as shown in Fig. 4(g, h, i), especially between the parental and drug resistant (IC_{50}) cell lines, which was obviously different from the results of LC-MS/MS. It possibly resulted from that WB was a semi-quantitative method depending on the antigen-antibody reaction and was possessed of a high detection limit [23]. The cross reaction was prone to appear, especially with the existence of homology metabolic enzymes. In addition, modification and structure change of gene transcription proteins were found after translation, thus the results were hard to reproduce [23–26].

In those studies of BCRP overexpression, the amount of BCRP in resistance cell lines was detected and compared with that in parental

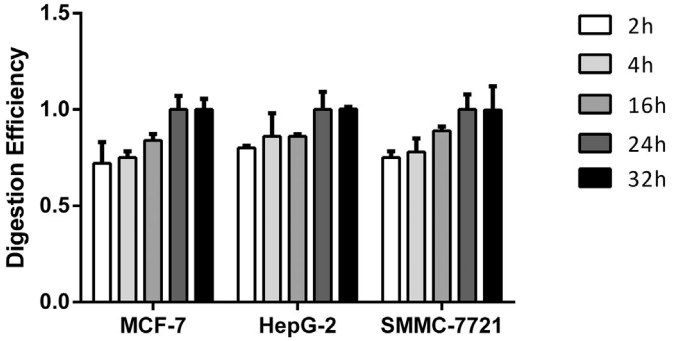

Fig. 3. The digestion efficiency of BCRP with the change of time in different cell lines (n = 5).

cells. In order to study protein expression or multidrug resistance properties more clearly, drug resistance levels were generally very high (hundreds of times), which was far beyond the actual clinical drug resistance degree [30]. However, the expression pattern of protein was variant under high and low levels of drug resistance, which resulted in the difference between the laboratory outcome and clinical practice eventually. Unfortunately, the detection limit of conventional analytical methods cannot meet the requirement of protein expression under low drug resistance level. The amounts of BCRP obtained by LC-MS/MS-based method were accurate and reliable.

In summary, the LC-MS/MS-based method developed and validated in this study was able to monitor the slight increase of BCRP expression more accurately and sensitively than RT-PCR and WB. Then, the existence of contradiction in existing literature is indeed due to the inaccuracy of the conventional quantitative method.

3.6. Comparison between the current and previous LC-MS/MS-based analytical methods

There are several studies on the detection of BCRP by mass spectrometry, as shown in Table 5. While exhibiting improved selectivity, sensitivity, and reproducibility compared with conventional methods, there were some limitations with the reported methods. One limitation was that a long run time was often required (for several transporters). The shortest run time was 4 min, while the precision of this method could not meet the requirements of the current biological guideline. Another limitation is that greater assay sensitivity might still be needed to determine the expression of BCRP with low abundance.

4. Conclusions

In our study, a new LC-MS/MS-based targeted proteomics method for the quantification of BCRP was developed by measuring its surrogate peptide. The method was proved to possess sufficient specificity, sensitivity, accuracy, precision and stability to measure BCRP in the drug resistant cell lines. Within the method, the amounts of target

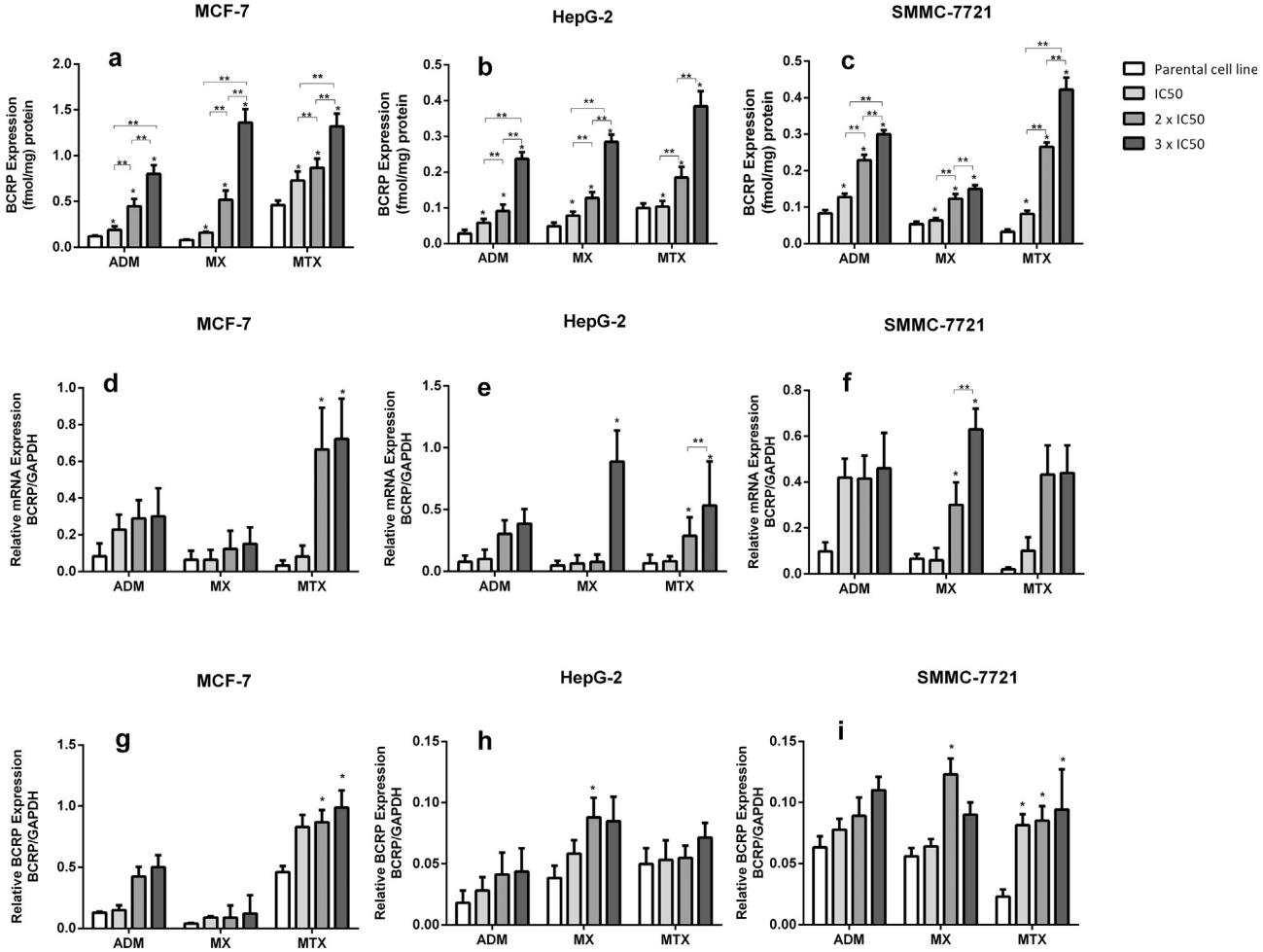

Fig. 4. Comparative analysis of BCRP expression and its mRNA expression in parental and different drug resistant cell lines detected by LC-MS/MS (a, b, c), RT-PCR (d, e, f) and WB (g, h, i), respectively. Data are given in mean ± SEM. ⋆ indicates significant differences between parental cell lines and drug resistant cell lines; ⋆⋆ indicates significant differences between drug resistant cell lines.

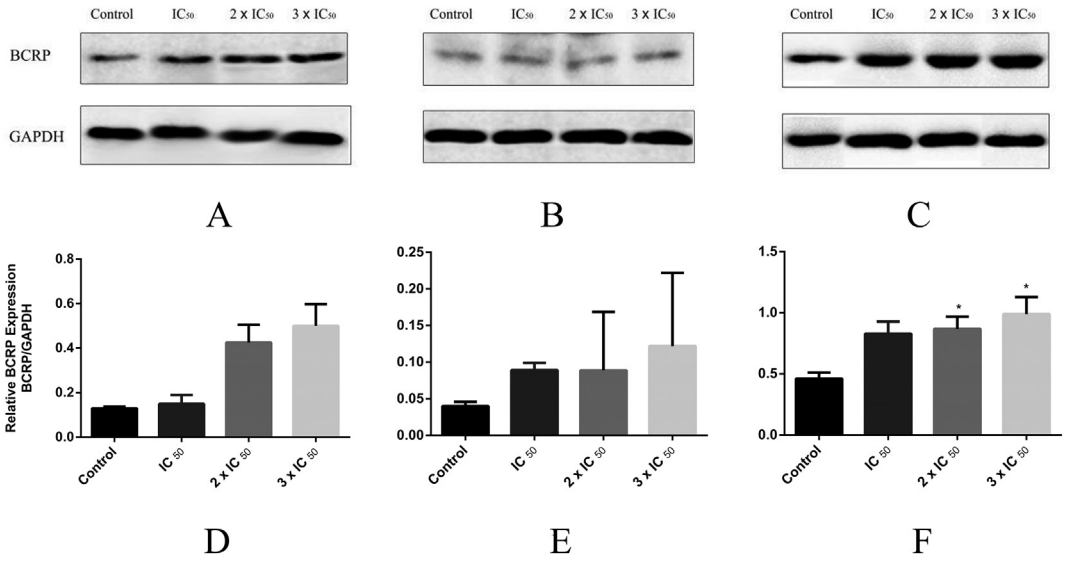

Fig. 5. The result of BCRP expression in MCF-7 monitored by Western Blot; the control is the parental cell line (A, D are the result of MCF-7 treated by ADM; B, E are the result of MCF-7 treated by MX; C, F are the result of MCF-7 treated by MTX, ⋆ indicates significant differences between parental cell lines and drug resistant cell lines).

Table 5
A comparison between this and previous LC–MS/MS-based methods on quantification of BCRP.

References	Surrogate peptide	Run time (min)	Linear range	Correlation coefficient (R^2)	LLOQ	Precision (RSD, %) or (CV, %)
This study	SSLLDVLAAR	5	0.096–96 fM	0.9982–0.995	0.096 fM	1.73–8.1
Harwood [21]	VIQELGLDK	> 40	100-fold	0.980	0.2 fM	2.3–8.0
Li [20]	SSLLDVLAAR	4	0.298–153 fM	0.995	0.030 nM	28%
Collins [14]	SSLLDVLAAR	30	2.76–88 fM	0.995	6.41 fM	< 20%
Gröer [27]	SSLLDVLAAR	25	0.1–25 nM	0.9838	0.1 nM	< 15% (< 20% at LLOQ)
Uchida [28]	SSLLDVLAAR	15	121.7–1947.7 atmol		60.9 atmol/μg	

proteins in drug resistant cells induced by different concentrations of ADM, MX, MTX, were successfully measured. Compared to the conventional analytical methods that provided only the ratios of BCRP amounts, the LC-MS/MS technique could monitor the protein expression more accurately in a low drug resistance level which was more similar to clinical practice.

Therefore, the LC-MS/MS-based method is confirmed to be superior to RT-PCR and WB and is advised to be used in the related researches to avoid the shortcomings of conventional analytical methods.

Declaration of Competing Interest

The authors declare no competing financial interest.

Acknowledgments

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