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A liquid chromatography–tandem mass spectrometry-based targeted proteomics assay for monitoring P-glycoprotein levels in human breast tissue

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ABSTRACT

Background: P-glycoprotein (P-gp) can efflux drugs from cancer cells, and its overexpression is commonly associated with multi-drug resistance (MDR). Thus, the accurate quantification of P-gp would help predict the response to chemotherapy and for prognosis of breast cancer patients.
Methods: An advanced liquid chromatography–tandem mass spectrometry (LC/MS/MS)-based targeted proteomics assay was developed and validated for monitoring P-gp levels in breast tissue.
Results: Tryptic peptide 368IIDNKPSIDSYSK380 was selected as a surrogate analyte for quantification, and immuno-depleted tissue extract was used as a surrogate matrix. Matched pairs of breast tissue samples from 60 patients who were suspected to have drug resistance were subject to analysis. The levels of P-gp were quantified. Using data from normal tissue, we suggested a P-gp reference interval. The experimental values of tumor tissue samples were compared with those obtained from Western blotting and immunohistochemistry (IHC). The result indicated that the targeted proteomics approach was comparable to IHC but provided a lower limit of quantification (LOQ) and could afford more reliable results at low concentrations than the other two methods.
Conclusions: LC/MS/MS-based targeted proteomics may allow the quantification of P-gp in breast tissue in a more accurate manner.

1. Introduction

The development of multi-drug resistance (MDR) remains a major clinical challenge in the current treatment of cancers, including breast cancer. This phenomenon is usually associated with an increased level of P-glycoprotein (P-gp), which can efflux the drugs out of cancer cells and thus decrease the intracellular drug accumulation in MDR cancers [1]. P-gp is encoded by the gene multi-drug resistance 1 (MDR1) and is normally expressed at low levels in breast tissue [2]. However, a number of studies have demonstrated a progressive development of P-gp overexpression in breast cancer patients during chemotherapy [3]. Thus, the accurate quantification of P-gp in clinical practice would help predict the individual response to chemotherapy and prognosis of breast cancer patients.

The level of P-gp in tissue samples has traditionally been measured using antibody-based methods, including Western blotting [4] and immunohistochemistry (IHC) [5]. While these techniques provide valuable information on protein levels, they often lack the necessary specificity and reproducibility [6]. More importantly, most of the assays that have been developed are qualitative or semi-quantitative but not quantitative [7]. Thus, other approaches are required to quantitatively monitor P-gp levels.

Mass spectrometry, perhaps most familiar for its use in discovery-based proteomics, can also be applied to quantify target proteins of interest (driven by hypothesis). In a targeted analysis, selected/multiple reaction monitoring (SRM or MRM) on a triple quadrupole instrument is generally employed for quantification, and liquid chromatography coupled on-line to tandem mass spectrometry (LC/MS/MS) assays are developed to detect fragment ion signals from proteolytic peptides representing the targeted protein [8,9]. The ion mass of the precursor peptide is set in the first mass analyzer (Q1), while a fragment peptide product ion, which is generated by collision-induced dissociation in Q2, is predefined in the third mass analyzer (Q3). The precursor/product ion *m/z* pair, referred to as the transition, is used to yield the chromatogram. The area under the curve of the chromatogram provides a quantitative measurement for each desired peptide and target protein. In this way, problems with detection specificity and antibody

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development in antibody-based methods are greatly minimized. Therefore, targeted proteomics is now considered a reliable alternative to other more ambiguous methods [10].

Our previous work has successfully determined P-gp in cultured breast cells using targeted proteomics [11]. In this report, we further applied this approach into clinical practice. An advanced LC/MS/MS-based targeted proteomics assay for the quantification of P-gp in breast tissue was developed and validated. This assay was then applied to the quantitative analysis of P-gp in 60 matched pairs of breast tissue samples from patients who were suspected to have drug resistance. The resulting values were compared to those obtained using Western blotting and IHC.

2. Materials and methods

2.1. Chemicals and reagents

The stable isotope-labeled amino acid was supplied by Cambridge Isotope Laboratories, Inc. The synthetic proteolytic peptide and its corresponding stable isotope-labeled internal standard were synthesized by ChinaPeptides Co., Ltd. Ammonium bicarbonate (NH_4HCO_3) was from Qiangshun Chemical Reagent Co., Ltd. DL-dithiothreitol (DTT), iodoacetamide (IAA), Tris–HCl and Triton X-114 were supplied by Sigma-Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) was obtained from Sinopharm Chemical Reagent Co. Sequencing grade modified trypsin was from Promega. Phosphate buffered saline (PBS) was from the Beyotime Institute of Biotechnology. Acetonitrile (ACN) and methanol were from Tedia Company, Inc. Trifluoroacetic acid (TFA) and formic acid (FA) were from Aladdin Chemistry Co., Ltd. and Xilong Chemical Industrial Factory Co., Ltd., respectively. Sodium dodecyl sulfate (SDS) was from Generay Biotech Co., Ltd. Water was purified and deionized with a Milli-Q system manufactured by Millipore.

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

A 1 mg/ml stock solution (purity ~99.3%) was prepared by weighing the peptide and dissolving it in deionized water. The solution was stored at −20 °C in a brown glass tube to protect it from light. An isotope-labeled synthetic peptide was used as an internal standard. The internal standard was also weighed, and a 5 μg/ml stock solution was prepared in deionized water. A 1000 ng/ml internal standard solution was prepared by diluting the stock solution with an ACN:water mixture (50:50, v/v) containing 0.1% FA.

Because matrix complexity is a significant obstacle in the quantification of endogenous analytes for which a true blank is not available, P-gp depleted tissue extract [12] was employed as a matrix here. The experimental details are given in the Supplementary material. The calibration standards were prepared by serial dilution of the stock solution using P-gp depleted tissue extract. The concentrations of the calibration standards were 10, 25, 50, 100, 250, 400, 700 and 1000 ng/ml. The QC standards for the lower limit of quantification (LLOQ), low QC, mid-QC and high QC were prepared at 10, 30, 200 and 800 ng/ml, respectively, in the same matrix and frozen prior to use.

2.3. Tissue collection

Breast tissue collection in this study was approved by the Institutional Review Board of Nanjing Medical University. Sixty pairs of breast tissue samples consisting of tumors and adjacent sections from patients who had invasive breast cancer and were suspected to have drug resistance were collected consecutively between January 2011 and February 2012 at Nanjing Maternity and Child Health Care Hospital, Nanjing, China (mean patient age, 52.9 ± 7.8 years; age range, 35–61 years). For these patients, initial active chemotherapy was demonstrated by reduced tumor volume, improved symptoms and decreased serological tumor markers. However, after a variable period, tumor not only was resistant to the agents treated, but also continued to develop resistance to combinations of non-cross-resistant regimens [13]. Tissue sections were confirmed as normal and cancerous by hospital pathologists. Among tumor tissues, 41 samples were invasive ductal carcinoma, 17 were invasive lobular carcinoma and 2 were other histological types. Tumor grade was assessed according to the Nottingham scheme [14]. Histological evaluation of adjacent normal tissue samples showed no indication of contamination from tumor or other abnormal cells. The patients were biologically unrelated, but all belonged to the Han Chinese ethnic group from the Jiangsu province in China. Informed consent was obtained from the subjects. Tissue samples were stored frozen at −80 °C until analysis. Prior to protein extraction, the tissue samples were thawed to room temperature and then rinsed thoroughly with deionized water. Fat tissue was removed and the remaining tissue was cut into small pieces and transferred to tubes.

2.4. Membrane protein extraction

Approximately 50 mg of tissue was weighed and resuspended in buffer containing 50 mmol/l Tris/HCl pH 7.4, 2 mmol/l EDTA, 1 mmol/l DTT, 150 mmol/l NaCl and 1% protease inhibitor cocktail. Samples were homogenized using a Bio-Gen PRO200 homogenizer (PRO Scientific Inc.). After centrifugation, the collected samples were resuspended in 500 μl of 1% Triton X-114 extraction buffer (1 mmol/l DTT and 2 mmol/l EDTA in 50 mmol/l Tris/HCl, pH 7.4) containing 1% protease inhibitor cocktail. The samples were incubated on ice for 30 min at 37 °C for 10 min, then centrifuged at 10,000 × *g* for 3 min to separate the detergent and aqueous phases. To achieve complete extraction, 500 μl of 1% Triton X-114 extraction buffer and 500 μl of 0.06% Triton X-114 wash buffer (1 mmol/l DTT and 2 mmol/l EDTA in 50 mmol/l Tris/HCl, pH 7.4) were added to the aqueous and detergent phases, respectively. The incubation and centrifugation steps were repeated. The detergent phases were combined, and the proteins were precipitated from cold acetone. The protein pellets were then dissolved in 1% SDS solution. The protein concentrations of the resulting membrane fractions were determined using a BCA protein assay kit (Pierce Biotechnology, Inc.).

2.5. In-solution tryptic digestion

A quantity of 100 μl of each sample (calibration standards, QCs and cell membrane fraction) was mixed with 50 μl of 50 mmol/l NH_4HCO_3. Denaturation was performed at 95 °C for 8 min. Subsequently, the protein was reduced by the addition of 50 mmol/l DTT until a final concentration of 10 mmol/l was achieved. The sample was then incubated at 60 °C for 20 min. The sample was then alkylated by adding 400 mmol/l IAA to obtain a final concentration of 50 mmol/l 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 to an Oasis HLB cartridge (60 mg/3 ml; Waters) that had been 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.

2.6. LC/MS/MS

An Agilent Series 1200 HPLC system (Agilent Technologies) and a 6410 Triple Quad LC/MS mass spectrometer (Agilent) were used for the LC/MS/MS studies.

The liquid chromatography separations were performed on a Hypersil GOLD column (3 μm, 20 mmol/l × 2.1 mmol/l; Thermo Fisher Scientific) 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 45 psi. The data were collected and processed using the Agilent MassHunter Workstation Software (ver B.01.04).

2.7. Conventional analytical methods

For the experimental details of IHC and Western blotting, please see the Supplementary material.

3. Results and discussion

3.1. Selection of surrogate peptide

The most critical step in the experimental design and assay establishment is the selection of proteolytic peptides that represent a candidate protein and that can provide specificity and an adequate response. To date, several LC/MS/MS-based targeted proteomics assays for the quantification of specific membrane transporters have been developed [15–18], whereas few studies have been conducted with tissue samples. The utility of peptides in these MRM assays should be considered carefully because the experimental methods, sample conditions and MS instrumentation used to obtain these spectra, are not always consistent [19]. For instance, the abundant tryptic peptides 817NTTGALTTR825 [20] and 262AGAVAEEVLAAIR270 [21] employed in the previous studies were not present in significant quantities here. Therefore, we searched the PeptideAtlas database (www.peptideatlas.org) for mass spectral evidence of P-gp [22]. Surrogate peptides were selected from the list of previously detected peptides using previously described selection criteria [15]. Then, we performed a LC/MS/MS analysis of tissue extract after SDS-PAGE separation and in-gel digestion (experimental procedures are shown in the Supplementary material) with the list of MRM transition pairs to determine the tryptic peptides that were present in the greatest abundance. The most abundant peptide was the triply charged ion of 368IIDNKPSIDSYSK380, rather than the 434STTVQLMQR442 that we previously observed in cell extract [11]. In addition, this sequence was found to be unique to P-gp (accession no. P08183 (MDR1_HUMAN), gi: 2506118) using a BLAST search, suggesting that it could be used to specifically quantify P-gp. The product ion spectrum of 368IIDNKPSIDSYSK380 is shown in Fig. 1A. The characteristic sequence-specific b ions and y ions were indicative of this peptide. Therefore, a synthetic stable isotope-labeled peptide, I*I*DNKPSIDSYSK, was prepared. The peptide was labeled at the isoleucine position ($^{13}C_6$) and eluted at the same retention time (5.2 min) as the non-labeled peptide (Fig. 1S).

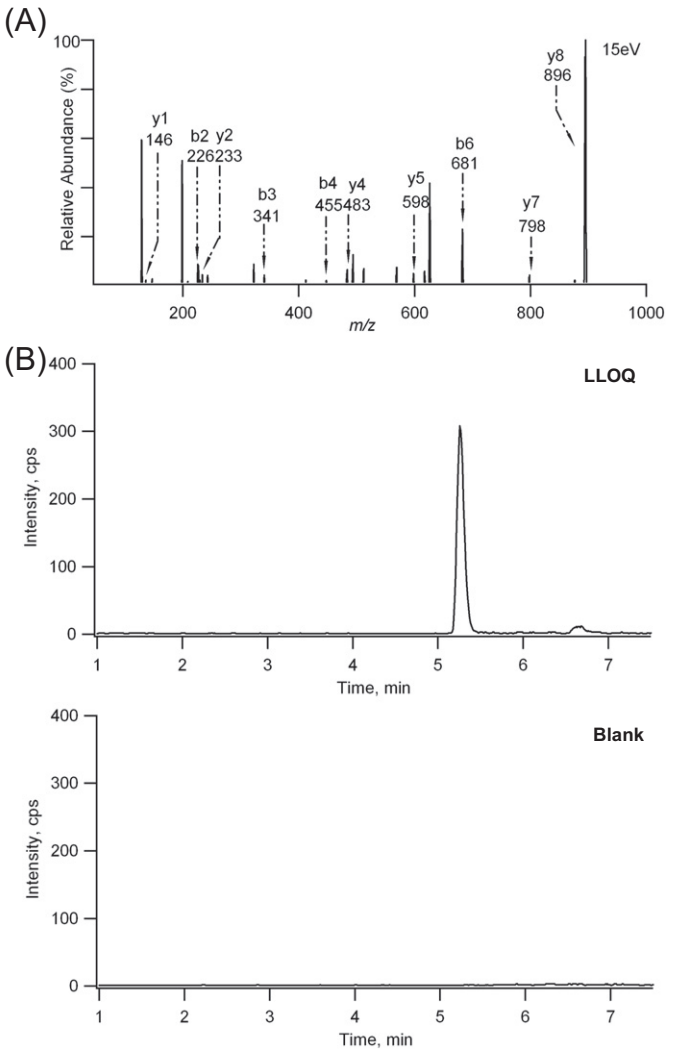

Fig. 1. The product ion spectrum of 368IIDNKPSIDSYSK380 (A) and the LC/MS/MS chromatograms of LLOQ and blank matrix (B). The internal standard is omitted for clarity.

3.2. Membrane protein extraction and digestion efficiency

The application of LC/MS/MS based targeted proteomics for the quantification of membrane proteins is usually hindered by the lack of pure protein standards for calibration [20]. Alternatively, a synthetic surrogate peptide can be spiked into a biological matrix and utilized as a standard to calibrate and quantify the membrane protein. In this case, the recovery of the membrane extraction and the completeness of the trypsin digestion must be carefully assessed.

As one of the most powerful tools for preparing membrane proteins [23], Triton X-114 was applied to extract P-gp from the breast tissue samples. Triton X-114 not only solubilizes membrane proteins but also has the ability to separate them from hydrophilic proteins *via* phase partitioning at physiological temperatures [24]. While Triton X-114 has often been employed for the extraction of membrane proteins from cultured cells, its application to tissue samples has been reported only rarely [25,26]. We therefore evaluated the utility of phase partitioning by comparing protein yields with those obtained using other detergents (*e.g.*, RIPA (Triton X-100)). As shown in Fig. 2S, a significantly higher yield of P-gp was obtained using Triton X-114. After two incubation and extraction steps, no detectable P-gp was observed in the tissue samples using Western blotting and the LC/MS/MS-based targeted proteomics assay (data not shown).

Insufficient digestion is another confounding factor in the absence of pure target protein and when detecting proteolytic peptides as an alternative to the target protein. Generally, extended surrogate peptides that contain a small number of amino acids from the surrounding sequence are employed to investigate the digestion efficiency. In this study, a substrate peptide, 366FKIIDNKPSIDSYSKSG382, containing 368IIDNKPSIDSYSK380 was synthesized. The substrate peptide was spiked into immuno-depleted matrix and digested using the same digestion protocol as that described for P-gp in the Experimental section. Both the undigested substrate peptide and the peptides after digestion were monitored using LC/MS/MS. Fig. 3S shows the absence of the starting substrate peptide, 366FKIIDNKPSIDSYSKSG382, and the presence of the product tryptic fragment, 368IIDNKPSIDSYSK380. The recovery of 368IIDNKPSIDSYSK380 was calculated by comparing the response ratios of the tryptic peptide after digestion and the equimolar surrogate

synthetic peptide standard in the digestion. Consequently, the estimated recovery was ~95.7%.

Notably, the quantification of proteins performed here using external synthetic peptide standards, strictly speaking, does not represent "absolute quantification," even after the optimization of the membrane protein extraction and digestion [27]. Ideally, pure target proteins of known concentration are utilized to control the levels of protein loss (or enrichment), extraction, denaturation and digestion [28]. The unavailability of target proteins for evaluation will subject the results to additional errors. For instance, the approach to determine the digestion efficiency does not consider the steric hindrance of enzymatic digestion, where the catalytic site may be buried within a large protein structure [29]. In addition, differences in P-gp extraction by various detergents were shown above. Nevertheless, the optimization of these preparation conditions can improve the quantitative analysis in a more accurate manner.

3.3. LC/MS/MS assay development and validation

MRM assays are generally developed on a triple quadrupole instrument to detect the fragment ion signals arising from unique surrogate peptides. In this study, the y8 ion, m/z 896.4, and the b6 ion, m/z 680.8, exhibited intense signals in the product ion spectrum of 368IIDNKPSIDSYSK380. Using the transitions of m/z 493.7 → 896.4 and m/z 497.7 → 896.4 (internal standard), a P-gp LC/MS/MS assay was developed. The m/z 493.7 → 680.8 was also evaluated here to serve as a confirmation of transition specificity. Method validation involved evaluating the linear range, accuracy, precision, limit of quantification (LOQ), recovery and stability.

Calibration and QC samples are generally prepared using an analyte-free matrix, whereas the quantification of P-gp is complicated by its endogenous presence. Because the matrix is preferred to represent the regular membrane protein component in tissue extracts, we employed P-gp depleted membrane fractions from pooled tissue samples as a surrogate matrix in this study. The level of depletion was significant in the immuno-depleted tissue extract, as determined by Western blotting and an LC/MS/MS-based targeted proteomics assay (Fig. 4S). In addition, a procedure of standard addition was used to assess the surrogate matrix [12]. The theoretical concentration of the P-gp extrapolated from the standard addition was compared to the concentration interpolated from the P-gp depleted tissue extract calibration curve. Agreement between these independent determinations was confirmed (Fig. 5S). Thus, immuno-depleted matrix can be used for the creation of calibration curves.

The calibration curve was constructed using a weighted linear regression model with a weighting factor of $1/x^2$. The relative peak area ratio of the analyte, 368IIDNKPSIDSYSK380, and the stable isotope-labeled internal standard was plotted as a function of concentration. The LOQ was 10 ng/ml. A representative chromatogram of the LLOQ is shown in Fig. 1B. Its response was >5 times the response of the blank matrix [30]. Moreover, the calculated values for pairs of MRM transitions (m/z 896.4/m/z 680.8) were consistent throughout the calibration range, demonstrating the specificity of the transitions.

The precision and accuracy of the assay were assessed by observing the response of the QC samples with four different concentrations of P-gp 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 average calculated concentrations to their nominal values (%bias). The results are listed in Table 1. Both the accuracy and precision were ≤±15% (LLOQ, ≤±20%) [30]. The QC data indicated acceptable accuracy and precision.

The recovery of the surrogate peptide was assessed using known amounts of peptide added to the P-gp depleted tissue extract. The percentage recovery was calculated as ((final concentration − initial concentration) / added concentration) × 100 [31]. The result was acceptable (within the range of 85–115%, Table 1S). The three cycle freeze-thaw, 48 h post-preparative (4 °C) and 12 h room temperature stabilities were also determined here. The results indicated that the stability of the peptide was acceptable (Table 2S).

Table 1
Accuracy and precision for the QC samples.

Nominal concentration	10 ng/ml	30 ng/ml	200 ng/ml	800 ng/ml
Mean	9.49	28.2	198	817
%bias	−5.1	−5.9	−1.2	2.1
Intra-day precision (%CV)	3.0	1.9	1.9	0.7
Inter-day precision (%CV)	0.6	1.5	3.0	2.3
n	18	18	18	18
Number of runs	3	3	3	3

3.4. Quantification of P-gp in breast tissue samples

Using the LC/MS/MS based targeted proteomics assay, 60 matched pairs of breast tissue samples were subjected to analysis. The levels of P-gp were accurately quantified as 0.092 ± 0.018 ng/mg (range: 0.058–0.142 ng/mg breast tissue) in the normal tissue and 0.136 ± 0.429 ng/mg (range: 0.056–1.48 ng/mg) in the tumor tissue (Fig. 2). Their difference was not statistically significant at a 95% confidence level. There was a large standard deviation in the results of the tumor samples, indicating of a more diverse population. Indeed, the samples were randomly selected from subjects suspected to have drug resistance. As previously reported, a variety of pathways have been suggested for cancer cells to acquire drug resistance [32]. Two major pathways include drug efflux and the direct suppression of apoptosis. Drug efflux occurs due to the increased membrane accumulation of various ATP-binding cassette (ABC) transporters, including P-gp. 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, such as Bcl-2. Therefore, the patients who were suspected to be drug resistant could have exhibited apoptosis suppression or drug efflux by ABC transporters other than P-gp. Furthermore, the variability of acquired resistance may also account for the large standard deviation of the measured P-gp levels.

The P-gp reference interval was calculated as 0.057 (95% CI, 0.050–0.064) ng/mg to 0.130 (95% CI, 0.122–0.136) ng/mg using MedCalc® software ver 11.6.1 and data from normal tissues. Thus, 29 of 60 tumor samples contained P-gp levels exceeding this estimated reference interval. Their mean value was 0.663 ± 0.408 ng/mg, which was significantly different from that of normal tissue at a 95% confidence level (Fig. 2). Among these samples, the levels of P-gp were also compared according to the clinical histopathological features (*i.e.*, tumor size, tumor grade, and lymph node status) of invasive ductal carcinoma (Table 3S and Fig. 6S). However, their differences were

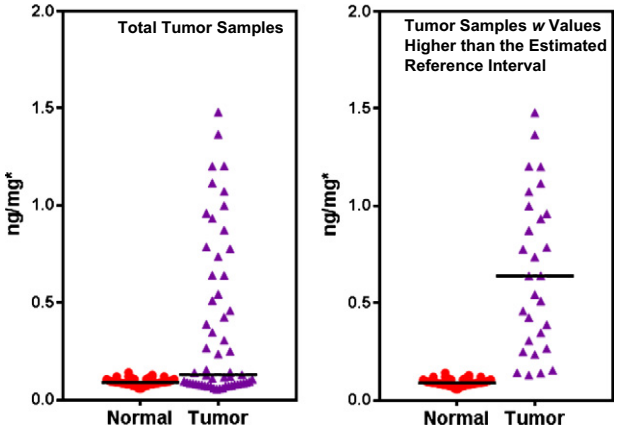

Fig. 2. P-gp amounts in 36 matched pairs of breast tissue samples before and after removal of tumor samples with P-gp level in the estimated reference interval.

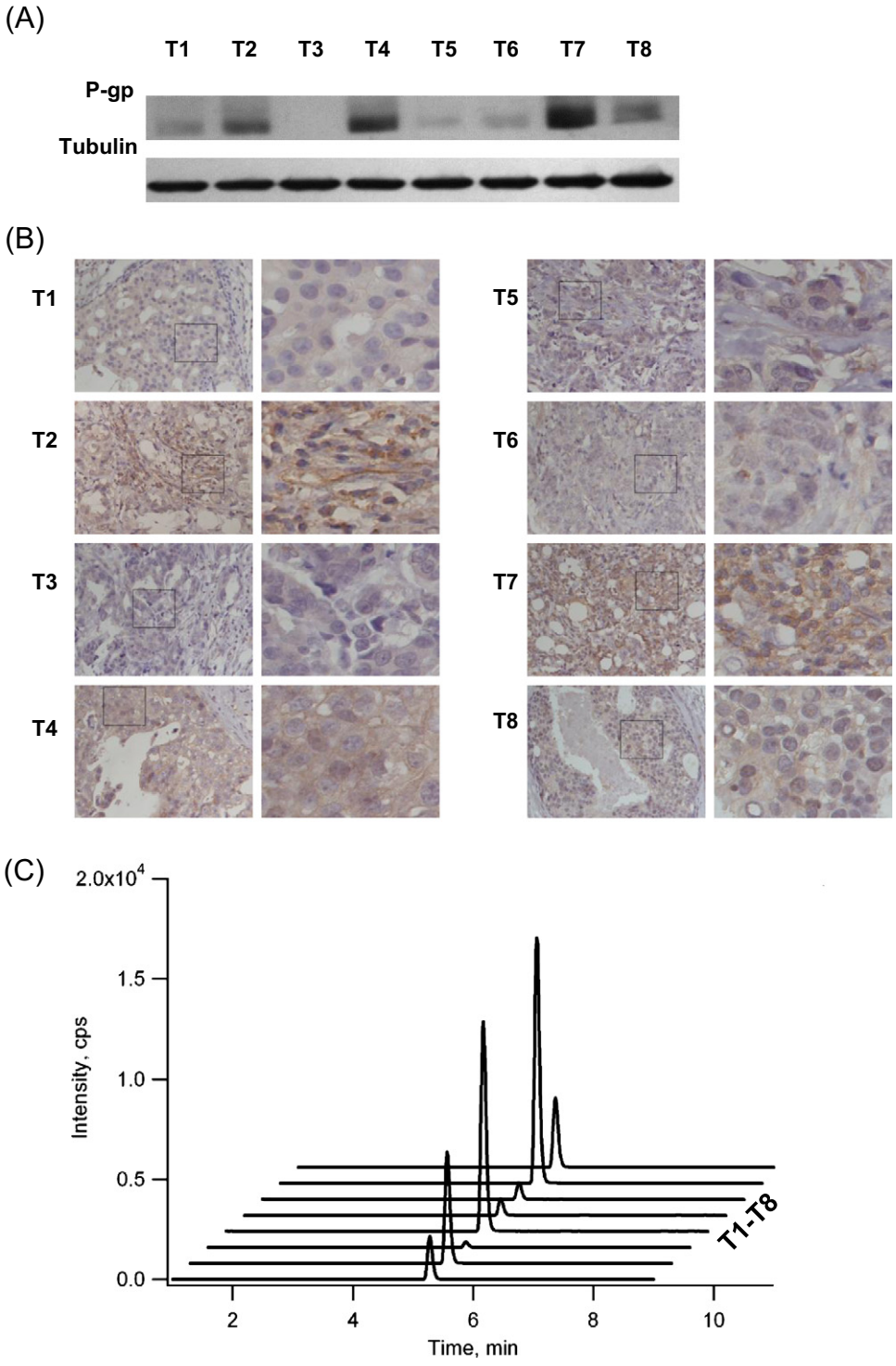

Fig. 3. Representative images of Western blotting (A), IHC (B) and LC/MS/MS-based targeted proteomics (C, 8 representative tumor tissue samples). Western blott analysis of P-gp is normalized with β-tubulin. Magnified regions of IHC images are also shown on the right.

not statistically significant ($p > 0.05$), probably due to the joint effect of other drug resistance mechanisms and a limited sample size in this study.

Because the level of P-gp is conventionally determined using IHC and Western blotting, the tissue samples in this study were also examined using these techniques. Representative images from IHC, Western blotting and LC/MS/MS-based targeted proteomics are shown in Fig. 3. P-gp was almost undetected in normal tissue using IHC and Western blotting. Of 60 tumor tissue samples, 26 showed detectable P-gp in IHC and 15 indicated a band of P-gp in Western blotting. Thus, the targeted proteomics approach provided a lower LOQ compared to the other two methods. Although the immunostaining was not scored in IHC, the percent positive cells (%) were counted for comparison with LC/MS/MS [33]. As shown in Fig. 4, the LC/MS/MS method was comparable to IHC ($y = 0.0145x + 0.170$, no significant deviation from linearity ($p = 0.48$)) using Passing–Bablok regression analysis (MedCalc). The estimated confidence intervals of the slope and intercept were (0.0103–0.0179) and (0.0473–0.352), respectively.

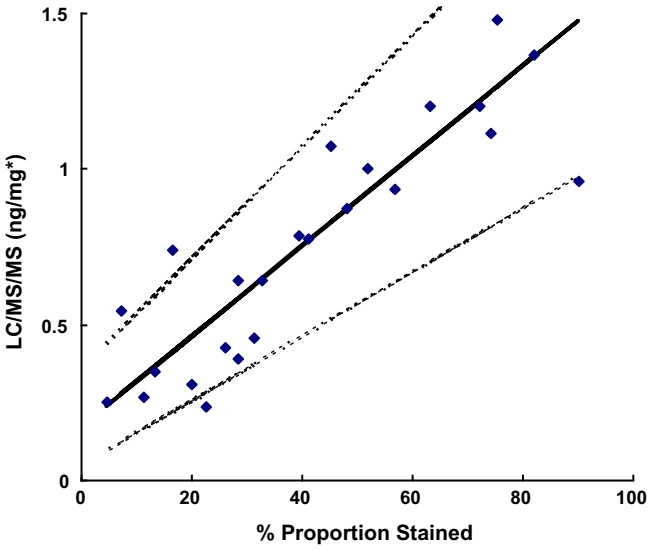

Fig. 4. Passing–Bablok regression analysis for the IHC *vs.* the LC/MS/MS assay. The solid line corresponds to the regression line. Dashed lines represent the 95% confidence interval for the regression line. * ng P-gp/mg breast tissue.

Nevertheless, there was a substantial disagreement between the results of these two methods in some samples. For instance, the sample with ~90% P-gp positive cells had a lower level of protein determined by the LC/MS/MS assay than the sample with ~50% positive cells. This disagreement could be due to the subjective positive counts in IHC. On the other hand, IHC is easy to perform and reproducible as a routine diagnostic tool in clinics [34]. One of its advantages over LC/MS/MS-based targeted proteomics is the capability of IHC to discern between tumors containing a small subpopulation of P-gp-positive cells and those showing a low level of P-gp in 100% of the cells [35]. Moreover, IHC can be used to determine the subcellular localization of proteins. Thus, targeted proteomics may be more suggestive of the diagnosis and treatment when combined with IHC in current situation. Finally, it deserves mention that commonly used prognostic scoring systems such as Nottingham Prognostic Index (NPI) [36] were not performed in this study, due to a limited sample size [34]. Thus, further careful comparison using large sample sizes will be required to illustrate the eligibility of the targeted proteomics assay.

4. Conclusions

The results presented in this study suggest that our LC/MS/MS-based targeted proteomics assay can allow the quantification of P-gp levels in breast tissue. Compared to Western blotting and IHC, this technique can more accurately monitor protein amounts. So far, no reference interval has been officially established for P-gp in clinical practice. Using the data of normal tissue, we suggested a P-gp reference interval and detected the overexpression of P-gp in some patients. Although the reference interval was determined using a limited number of individuals, this study demonstrates that the targeted proteomics approach can potentially detect P-gp mediated drug resistance. Future enlargement of the sample size and large reference interval studies may be required. These efforts may contribute to the improvement of diagnosis and the treatment of breast cancer patients with drug resistance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2014.06.013.

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