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Quantitative assessment of serum heat shock protein 27 for the diagnosis of epithelial ovarian cancer using targeted proteomics coupled with immunoaffinity enrichment

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ABSTRACT

Background: Heat shock protein 27 (HSP27) may take part in the epithelial ovarian cancer (EOC) malignant process because it is elevated in the serum of EOC patients, suggesting that HSP27 may serve as an EOC biomarker to complement the standard serum carbohydrate antigen 125 (CA125) test. Thus, accurate quantification of serum HSP27 would assist the diagnosis of EOC.

Methods: Liquid chromatography-tandem mass spectrometry (LC–MS/MS)-based targeted proteomics coupled with an immunoaffinity enrichment assay was developed and validated to monitor HSP27 concentrations in serum.

Results: Tryptic peptide 80QLSSGVSEIR89 was selected as a surrogate analyte for quantification, and an immuno-depleted serum extract was used as a surrogate matrix. Immunoaffinity enrichment was effective for protein enrichment and sensitivity enhancement, and the resulting LOQ was 500 pg/ml (> 10-fold increase). Then, serum HSP27 concentrations in EOC patients, benign ovarian tumors patients and healthy volunteers were accurately determined to be 4.95 ± 0.37 ng/ml, 2.98 ± 0.16 ng/ml and 2.82 ± 0.15 ng/ml, respectively, suggesting that the EOC samples had significantly higher concentrations of HSP27 than a sample from benign ovarian tumor patients. The experimental values for the samples were compared with those obtained from enzyme-linked immune sorbent assays (ELISAs). The ROC curve analysis showed that the combined area under the curve (AUC) for CA125 and HSP27 was 0.88, which is significantly superior to that of CA125 alone.

Conclusions: Targeted proteomics coupled with immunoaffinity enrichment may provide more accurate quantification of low-abundant proteins.

1. Introduction

Each year, more than several hundred thousand patients are diagnosed with ovarian cancer, a disease that contributes to > 2 hundred thousand deaths annually worldwide [1,2]. Ovarian cancer is first among the gynecologic malignancies and has the highest mortality rate [1,3]. Ovarian cancer is also the leading cause of cancer-related deaths in women in China [4]. Epithelial ovarian cancer (EOC) accounts for approximately 90% of all ovarian cancer cases and is often metastatic at the time of diagnosis because of its asymptomatic nature. Peritoneal metastasis is one of the main causes of death in EOC, while the 5-y survival rate is just 46% among all populations [5,6]. Therefore, early detection of EOC is vital.

Currently, detection of serum carbohydrate antigen 125 (CA125) is the mainly applicable method for early diagnosis of EOC [7,8]. However, it has been widely accepted that detection of EOC based on the

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single biomarker of CA125 may not provide optimal diagnostic accuracy because it is only increased in approximately 50% of stage I and 70–90% of advanced EOC cases, leading to high false positive rates [9,10]. Consequently, a novel circulating serum biomarker for the detection of ovarian cancer and multiple biomarker tests performed on each individual are urgently needed [11,12]. Heat shock protein 27 (HSP27) is a member of the heat shock protein family, the names of which are based on their molecular weight [13]. It has been reported that HSP27 expression is involved in cancer cell differentiation, development, apoptosis and tumorigenicity [14,15]. Recent evidence demonstrated that HSP27 may also take part in the EOC malignant process. EOCs have been demonstrated to express higher concentrations of HSP27 than those of benign tumors [16], and increased HSP27 concentrations are associated with a high grade and reduced survival in EOC [17]. Moreover, a recent study suggested that serum HSP27 concentrations are elevated in patients with EOC [18]. Specifically, the concentration of HSP27 in tissue or serum may be applied as a potential additional biomarker for EOC. Tissues that could represent true expression during the onset and progression of cancer are the best source of material to assay early diagnostic biomarkers. However, invasive sampling procedures that may harm or kill an organism under investigation limit such applications. In recent years, interest in the identification and description of cancer by noninvasive surrogate markers has been increasing [19,20]. The advantages of biomarkers in serum include the following: (a) can detect missing invasion; (b) can be repeatedly checked; (c) may be performed in ambulatory settings; and (d) are applicable for monitoring disease progression. However, a prerequisite for the application of serum biomarkers in clinical serum practice is to accurately profile their expression.

Several commercial assays are widely used for the measurement of serum HSP27 in vitro. Most of these assays are enzyme-linked immune sorbent assays (ELISAs) based on antibodies against HSP27. While this technique provides valuable information on the protein concentrations and can be extremely sensitive, its limitations are also widely recognized. As the name suggests, the ELISA method requires protein horseradish peroxidase (HRP) enzymes that act as an amplifier. The HRP enzymes are sensitive to reaction conditions including time, temperature and pH, which restrict the universal application of the enzyme-based amplified technique [21]. This limitation may lead to a false positive result, showing that HSP27 may also be elevated under normal physiological conditions to some extent. Additionally, the results from commercial clinical HSP27 ELISAs vary from each other by the types of antibodies and calibrators, resulting in considerable measurement variability [22,23]. Thus, liquid chromatography-tandem mass spectrometry (LC–MS/MS)-based targeted proteomics, which allows researchers to quantify proteins with quantitative accuracy, reproducibility and wide dynamic ranges, is an alternative technique. The underlying principle of this targeted proteomics approach is the specific measurement of a protein of interest using surrogate peptides. In a targeted proteomics analysis, multiple/selected reaction monitoring (MRM or SRM) on a triple quadrupole instrument is generally employed for detecting surrogate peptides that are generated from the target protein in protease digestion [24]. Then, quantification is accomplished by comparing the summed value of the surrogate peptide(s) to that of the corresponding stable isotope-labeled peptide(s). In our previous study, we successfully detected HSP27 amounts in cultured breast cancer cells using targeted proteomics [25]. High concentrations of interfering substances are, however, generally the main issue in clinical serum practice because most biomarkers are at low concentrations. Despite this issue, targeted proteomics has recently become increasingly popular. Therefore, extensive sample preparation should be performed in advance, such as immunoaffinity enrichment.

2. Materials and methods

2.1. Chemicals and reagents

The synthetic peptides and corresponding internal standard peptides containing stable isotope-labeled amino acids were developed by ChinaPeptides Co., Ltd. The purities of the peptides were also provided by the manufacturer. DL-dithiothreitol (DTT) and iodoacetamide (IAA) were purchased from Sigma-Aldrich. Acetonitrile (ACN) and methanol were provided by Tedia Company, Inc. Sequencing grade-modified trypsin was obtained from Promega Co., Ltd. Trifluoroacetic acid (TFA) and formic acid (FA) were provided by Sigma-Aldrich Co., Ltd. and Xilong Chemical Industrial Factory Co., Ltd., respectively. The HSP27 antibody, HSP27 ELISA assay, and protein A/G-agarose were from Abcam, Multisciences and Abmart, respectively. The recombination HSP27 protein was supplied by Novus Biologicals.

2.2. Sample collection

In this study, 42 EOC patients and thirty-six benign ovarian tumor serum samples were obtained with informed consent from patients consecutively between September 2015 and December 2017 at Nanjing Maternity and Child Health Care Hospital and First Affiliated Hospital of Nanjing Medical University. The methods were conducted in accordance with the approved guidelines. The patients were biologically unrelated, but all belonged to the Han Chinese ethnic group from Jiangsu Province in China. Additionally, 36 serum samples from healthy volunteers were collected. Serum sample collection was approved by the Institutional Review Board of Nanjing Medical University. All patients were informed in advance, and signed explicit informed consent.

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

Recombinant human HSP27 protein stock solutions (1 mg/ml) were purchased and prepared. The solutions were stored at −80 °C in a tube, and we avoided repeated freezing and thawing to protect them from degradation. In this report, 1 μg/ml stock isotope-labeled synthetic peptide solutions were prepared in deionized water. Thereafter, a final concentration of 2.5 ng/ml for the internal standard peptides was prepared by diluting the stock solution into a mixture of ACN:water (50:50, *v*/v) containing 0.1% FA. Calibration standards were prepared at 0.5, 1, 2.5, 5, 10, 25 and 50 ng and then diluted and digested in 1 ml of pooled serum. QC standards, i.e., the lower limit of quantification (LLOQ), including low QC, mid QC and high QC standards, were prepared at 0.5, 1.5, 5 and 40 ng/ml, respectively, in the same matrix and frozen prior to use.

2.4. Protein immunoaffinity enrichment and in-solution tryptic digestion

Then, 1 ml serum was incubated with rabbit polyclonal HSP27 antibody overnight at 4 °C on a rocker, following by capture by protein A/G-agarose at 4 °C for 4 h. Then, the sample was centrifuged at 3000 × *g* for 5 min and washed 3 times with RIPA buffer to remove non-specifically bound proteins. After enrichment, 50 μl of 50 mmol/l NH_4HCO_3 was added to 100 μl of each sample. Then, the samples were denatured at 95 °C for 8 min. Subsequently, the protein was reduced with the addition of 50 mmol/l DTT and incubated at 60 °C for 20 min at a final concentration of 10 mmol/l. Then, 400 mmol/l IAA was added to obtain a final concentration of 50 mmol/l to alkylate the sample and incubated for 6 h at room temperature in the dark. The sample was digested via the addition of 4 μg of sequencing grade trypsin at 37 °C for 48 h. Then, 10 μl of 0.1% TFA was added to stop the reaction. Afterward, the internal standard solution was added to the mixture sample to obtain a final concentration of 5 ng/ml before transferring it

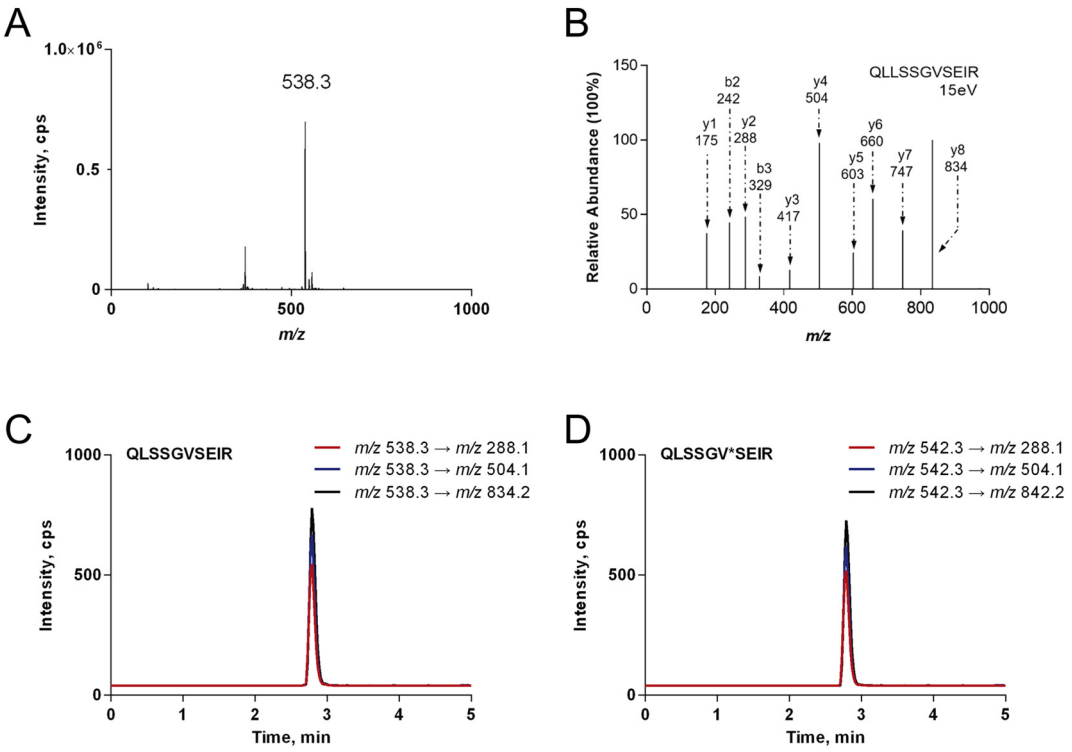

Fig. 1. The parent ion spectrum (A) and product ion spectrum of 80QLSSGVSEIR89 (B). LC–MS/MS chromatograms of equimolar 80QLSSGVSEIR89 (C) and the stable isotope-labeled internal standard (D). The MRM transitions of *m/z* 538.3 → 288.1, *m/z* 538.3 → 504.1 and *m/z* 538.3 → 834.2 and *m/z* 542.3 → 288.1 and *m/z* 542.3 → 288.1, *m/z* 542.3 → 504.1 and *m/z* 542.3 → 842.2 (internal standard) were used.

into an Oasis HLB cartridge (60 mg/3 ml; Waters), which had been preconditioned with a mixture of ACN:water. After loading the samples, the cartridge was washed with 2 ml of water and 2 ml of ACN:water (50:50, *v*/v) and eluted with 1 ml of 100% ACN. Finally, the eluent was evaporated to dryness, and the sample was resuspended in 100 µl of ACN:water (50:50, v/v) containing 0.1% FA.

2.5. LC–MS/MS analysis

An Agilent Series 1290 UPLC system and a 6460 triple quad LC–MS mass spectrometer were used for LC–MS/MS analysis. The liquid chromatography separation was performed on an Agilent SB C18 (2.7 µm, 30 mm × 2.1 mm, Agilent, USA) at room temperature. Solvent A (0.1% FA in water) and solvent B (0.1% FA in methanol) were used as mobile phases with a flow rate of 0.3 ml/min. The linear gradient 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 5 µl.

The mass spectrometer was interfaced with an electrospray ion source, and the positive MRM mode was operated. Q1 and Q3 were both set at unit resolution. The temperature of the drying gas was held at 350 °C, and its flow rate was 10 l/min. The nebulizer pressure was optimized to 35 psi, while the electrospray capillary voltage was set to 4000 V. Data were collected and analyzed using Agilent MassHunter Workstation Software (ver. B.06.00). Method validation included the evaluation of the linear range, LOQ, stability, accuracy and precision. The acceptance criteria used for validation and the detailed procedures have been described in several publications [26,27].

2.6. Comparative study

A commercially available ELISA assay was used to compare with the results obtained from our assay. First, 100 µl of assay diluent was added to each well, followed by 20 µl of the standards and samples. The plate was then sealed with seal film, kept at room temperature for 1 h and washed with 400 µl of wash buffer 3 times. Afterward, 100 µl of conjugate solution was added to each well, and they were incubated at room temperature for 1 h. After washing, 100 µl of substrate solution was added and incubated at room temperature for 30 min in the dark. Finally, 100 µl of stop solution was added, and the optical density of each well was read at 450 nm.

2.7. Data analysis

SPSS statistical software was used for the normal distribution and homogeneity of variance test. Student's *t*-test (2 tails) and 1-way ANOVA were used to analyze the difference between the normal distribution and homogeneity of the variance groups, while the rank sum test was applied for the abnormal distribution or heterogeneity of variance data. Receiver operating characteristic (ROC) curves were produced to discriminate EOC patients from the healthy volunteers and from the benign ovarian tumor patients. The optimal sensitivity and specificity from the ROC curves were determined via the standard method. Data are presented as Means ± S.D. The general acceptance concentration of significance was $p < 0.05$.

3. Results and discussion

3.1. Selection of surrogate peptide

For establishment of the experimental design and assay, the most critical step is the selection of tryptic peptides that represent a candidate protein for a targeted analysis of proteins. The empirical rules have been extensively described in our previous work [25]. Briefly, the surrogate peptides should (a) not be found in any other proteins using a BLAST search, (b) provide specificity and an adequate mass response, (c) generate a high-quality MRM, and (d) be completely digested. Following our previous similar process and the results, the most abundant peptide in the mass spectrum was determined to be the doubly charged

ion of 80QLSSGVSEIR89 (Fig. 1A), which is in accordance with the result of ESP Predictor (0.84). A BLAST search was used to verify the specificity of the peptide, and it was found not to match any other protein. The product ion spectra and LC–MS/MS chromatograms of the reporter ions are shown in Fig. 1B. Thus, the response of the product ions and the corresponding 3 MRM transitions provided with the product ions were y2 at *m/z* 288.1, y4 at *m/z* 504.1, and y8 at *m/z* 834.2, which were abundant (Fig. 1C). Moreover, the MRM transitions provided with the product ions, y2, y4, and y8, gave the best signal-to-noise (S/N) and LOQ among the product ions. In addition, the corresponding stable isotope-labeled peptide was prepared to serve as an internal standard. In detail, isotope-labeled [D8]Val was placed into the peptide at position 6 to form QLSSGV*SEIR, which produced an 8 Da molecular mass shift from the nonlabeled peptide. The retention times were identical between the QLSSGVSEIR and its isotope-labeled peptide, QLSSGV*SEIR (~2.8 min; Fig. 1D). Thus, the peak areas from the 3 transitions at *m/z* 538.3 → 288.1, *m/z* 538.3 → 504.1 and *m/z* 538.3 → 834.2 of the surrogate peptide and at *m/z* 542.3 → 288.1, *m/z* 542.3 → 504.1 and *m/z* 542.3 → 842.2 of the internal peptide were summed. The summed value of the surrogate peptide was compared to the stable isotope-labeled internal standard to generate a relative peak area ratio, which was used to plot against the calibration curve concentration and sample quantitation. Following the similar process in our previous study, recombination HSP27 protein was incubated with sequencing grade-modified trypsin. The estimated value of digestion efficiency was obtained (96.4 ± 3.4%) by calculating the relative ratios of the equimolar concentration of the peptide and the recombination HSP27 protein (Fig. S1), which are consistent with the score value generated by the PeptideCutter software.

3.2. Protein immunoaffinity enrichment

A promising approach that employs affinity reagents, such as antibodies, for specific enrichment or isolation of target proteins has been widely used prior to mass spectrometry [28]. Thus, a commercial protein antibody was used in this study. In the first attempt, the interaction between protein A/G-agarose and human serum albumin was investigated because native protein G has 3 IgG binding domains and a site for human serum albumin [29]. Human serum (1 ml) was incubated with protein A/G-agarose, separated, and then detected by SDS-PAGE. The results indicated that there was no cross combination between the protein A/G-agarose and human serum albumin (Fig. S2) because the gene sequence coding for the albumin-binding site was eliminated in the process of recombinant protein A/G production. The effect of cross-linking the HSP27 and anti-HSP27 polyclonal antibody was then investigated. An increasing amount of antibody was incubated with 500 µl of serum spiked with a fixed amount of recombinant HSP27 (50 ng) in a fixed sample volume of 1 ml. As a result, no significant increase in the binding reaction was observed from the addition of 1 µg of antibody (Fig. S3). Moreover, the efficiency of the binding reaction showed that a temperature of 4 °C and an overnight reaction time (12*h*) were favorable (Fig. S3). After optimization of the reaction conditions, the results of the measurements indicated that an LLOQ of 500 pg/ml protein in pooled serum could be achieved using the anti-HSP27 polyclonal antibody, which was > 10-fold enhancement of sensitivity compared with that of directly targeted proteomics after serum depletion (Fig. S4).

3.3. Development and validation of a LC–MS/MS–based targeted proteomics assay for HSP27

In the present study, using the transitions of *m/z* 538.3 → 288.1, *m/z* 538.3 → 504.1 and *m/z* 538.3 → 834.2 and *m/z* 542.3 → 288.1, *m/z* 542.3 → 504.1 and *m/z* 542.3 → 842.2 (internal standard), we developed and validated an HSP27 LC–MS/MS assay. The calibration curve in pooled serum was constructed in a weighted linear regression model to determine the linearity. Previous evidence indicates that solid-phase extraction (SPE) is a promising technique for sample cleanup, and this technique was also used in this study for sample preparation [30]. The relative peak area ratio to compare the surrogate peptide QLSSGVSEIR to the stable isotope-labeled internal standard QLSSGV*SEIR was plotted against the concentration.

As described in our previous study, the matrix effect is another issue during targeted proteomics analysis [31]. Since a matrix is preferred to represent the regular protein component in biological samples, we employed the HSP27-depleted serum fraction as a surrogate matrix in this study. As shown, no significant interfering peak was found at the retention time of HSP27 in the chromatogram of the HSP27-depleted serum, and no response value was observed in the ELISA assay (Fig. S5). Then, the slopes of the calibration curves in the pure solvent matrix (i.e., deionized water) and in pooled serum were compared to assess the matrix effect. As illustrated in Fig. S6, the percentage of the difference between the slopes is positive in the case of signal enhancement, whereas a negative value is indicative of ion suppression from nonspecific adsorbent proteins. Thus, the calibration curve for the pooled serum matrix produced good linearity for the standard addition analysis (Fig. S7).

The precision and accuracy of the assay were assessed by observing the response of the QC samples with 4 different concentrations of HSP27 in 3 replicates. The intra- and inter-day precisions are shown as the percent coefficient of variation (%CV). The accuracy was obtained by comparing the averaged calculated concentrations to their nominal values (%bias). As shown in Table S1 and S2, both the accuracy and precision were ≤ ± 15% (LLOQ, ≤ ± 20%), and the recovery was between 80% and 120%. An evaluation of 3 freeze-thaw cycles, 48 h postpreparation (4 °C) and the 12 h room temperature stability was also performed. The results demonstrated acceptable stability of the peptides digested from the protein.

3.4. Quantitative analysis of HSP27 in EOC serum samples and method comparison

The LC–MS/MS-based targeted proteomics coupled with the immunoaffinity enrichment assay that was developed and validated above was first applied to the detection and quantification of HSP27 in human serum samples. Forty-2 samples from EOC patients, 36 benign ovarian tumor patients and 36 healthy volunteers were analyzed. As shown in Fig. 2, the concentrations of serum HSP27 were accurately quantified to be 2.82 ± 0.15 ng/ml (range: 1.55–4.36 ng/ml) in the normal samples, 2.98 ± 0.16 ng/ml (range: 1.25–5.30 ng/ml) in the benign ovarian tumor patient samples and 4.95 ± 0.37 ng/ml (range: 1.32–14.12 ng/ml) in the EOC samples. Interestingly, the HSP27 values were not significantly different between the benign ovarian tumor patients and healthy volunteers using QLSSGVSEIR as the surrogate peptide (P = NS). The rank sum test showed that the EOC samples had significantly higher concentrations of HSP27 than those of the benign ovarian tumor patients ($P < 0.001$) and healthy volunteers ($P < 0.001$). To further investigate whether the serum total HSP27 concentrations in the EOC patients were correlated with peritoneal metastasis, we divided the patients into peritoneal metastasis ($N = 20$) and without peritoneal metastasis ($N = 22$) groups. Fig. S8 result shows that the serum concentrations of HSP27 in samples from those with peritoneal metastasis (5.34 ± 0.56 ng/ml (range: 2.21–14.12 ng/ml)) were higher than in those from patients without peritoneal metastasis (4.57 ± 0.48 ng/ml (range: 1.32–10.21 ng/ml)). However, there was no correlation between the serum concentrations of HSP27 and the grade of EOC.

Additionally, we performed a ROC curve analysis to compare and evaluate the power of HSP27 to predict EOC. The samples were divided into EOC patient groups and non-EOC patient groups (benign ovarian tumor samples), whereas the value of serum CA125 was obtained from a clinical laboratory. The results showed that the ROC curve for CA125

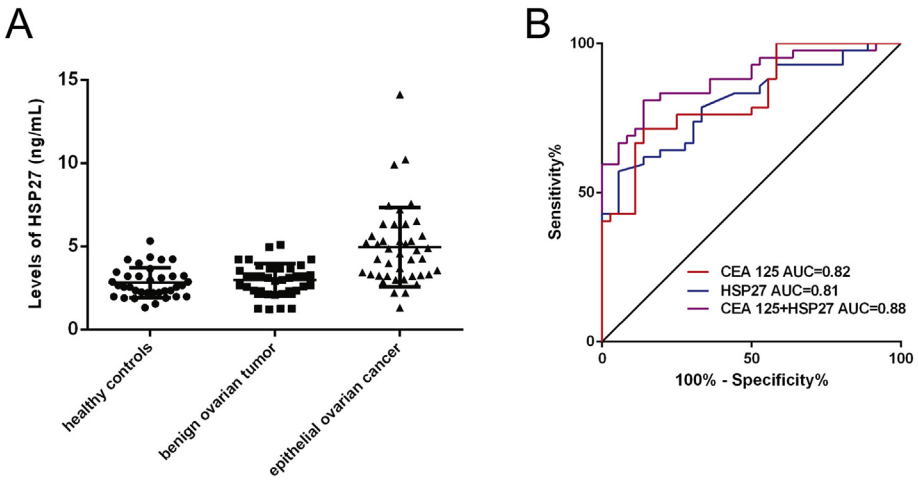

Fig. 2. Serum HSP27 concentrations in healthy volunteers, benign ovarian tumor patients and epithelial ovarian cancer patients (A), and ROC analysis of HSP27 and CA125 (B).

had an area under the curve (AUC) of 0.82 (95% CI = 0.73 to 0.91). The HSP27 AUC was 0.81 (95% CI = 0.71 to 0.90) (Fig. 2B). However, no significant difference was observed between these 2 markers because most of the CI values overlapped. Then, multivariate logistic regression analysis was performed for both markers combined. As Fig. 2B shows, the AUC of these 2 markers was 0.88 (95% CI = 0.81 to 0.96), which was significantly superior to that of CA125 alone. Our results suggested that serum HSP27 could provide the additional sensitivity and specificity required to improve the diagnostic and prognostic value of the CA125 test.

Subsequently, we compared our proteomics results to the conventional ELISA method values to evaluate the LC–MS/MS performance. Pearson correlation coefficient analysis for value comparison was performed using GraphPad Prism software version 7.0. Using the data points (benign ovarian tumor groups plus EOC groups, $N = 78$), the values from the LC–MS/MS method were well correlated to the ELISA assay (Y = 0.961X + 0.380), as shown in Fig. 3A. The estimated 95% confidence intervals of the Pearson correlation coefficient (R), the Y-intercept and the X-intercept were 0.887 to 0.953, −0.025 to 0.785 and −0.892 to 0.024, respectively. The average difference in serum concentrations detected by LC–MS/MS vs. ELISA was significant ($P < 0.05$, paired rank sum test) with a mean difference of 0.224 ng/ml and a 95% confidence interval of 0.038 to 0.410 ng/ml (Fig. 3B). The HSP27 values measured by ELISA were higher than those values obtained from LC–MS/MS (Fig. 3C).

4. Discussion and conclusions

In this study, we established an LC–MS/MS-based targeted proteomics method coupled with immunoaffinity enrichment for the quantification of HSP27 in human serum. This approach is a further application of our previous research on clinical noninvasive diagnosis.

It is well known that matrix effects can alter the ionization of target analytes and the chromatographic response of target analytes, leading

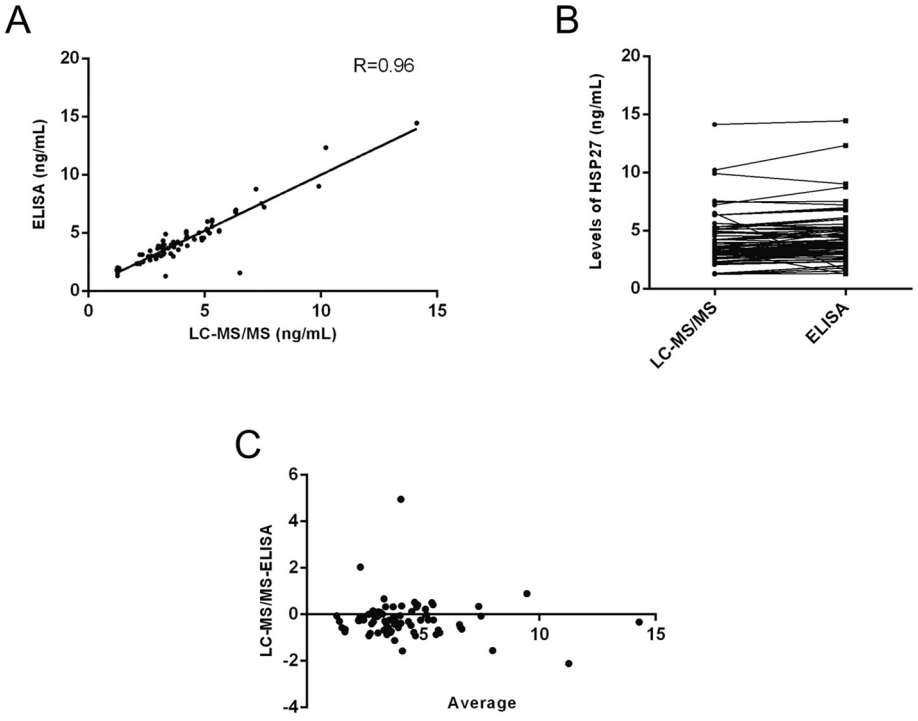

Fig. 3. Passing–Bablok regression analysis (A), rank sum test (B) and the corresponding Bland–Altman plot (C) for ELISA vs. targeted proteomics.

to reduced or increased sensitivity of the analysis. Therefore, sample preparation is a great challenge in targeted proteomics, especially for serum-based proteomics research [28]. Complex biological fluids, such as serum, contain thousands of large dynamic concentration ranges of proteins, from highly abundant proteins to extremely low-abundance proteins, spanning > 10 orders of magnitude. From a medical point of view, serum is the most complete informative substance and the richest proteome, containing 60–80 mg total protein per milliliter. Indeed, only a few proteins (e.g., albumin, transferrin, lipoproteins, and immunoglobulins) constitute 90% of the serum protein content. Prior studies have shown that twelve proteins make up 90% of this remaining 10% of serum protein content [32]. Unfortunately, only 1% of the entire serum protein content is made up of proteins that are in low abundance but are of great interest in the search for potential biomarkers in clinical trials [33]. Thus, the repeatability and sensitivity of many techniques in serum proteomics are significantly affected by these abundant proteins, leading to difficulties in the quantification of low-abundance proteins. Unfortunately, matters are complicated by the fact that potential candidate biomarkers in serum are often present in low concentrations [34].

Thus, several commercial affinity removal kits that are available have been developed to remove up to 20 major high-abundance proteins in serum. Although this strategy is highly specific, the remaining proteins after depletion are still sufficiently abundant, which limits the low-abundance protein detection. Moreover, evidence has been provided that there is a risk of losing low-abundance proteins after the use of affinity removal kits [35]. Our results also suggested that there was HSP27 loss to an unpredictable extent in high-abundance protein-depleted serum. Previous evidence has emerged that ion suppression by other serum proteins that generate peptides may lead to the low signal of the surrogate peptide [36]. In immunoaffinity enrichment based on the antibody approach, interferences caused by peptides generated from other endogenous serum proteins could be avoided because most endogenous proteins are removed with the help of an antibody [37].

During the HSP27 measurement using LC–MS/MS coupled with the immunoaffinity enrichment method, the assay demonstrated that antibody affinity enrichment was effective for protein enrichment and sensitivity enhancement, and a > 10-fold enhancement of sensitivity was achieved using this combination assay. This allowed the quantification of low-abundance HSP27 in real biological samples. Using this assay, EOC patients, benign ovarian tumors and the corresponding control samples were analyzed. The differential expression of serum HSP27 in EOC and in benign ovarian tumors that we obtained is consistent with other studies in tissue [17] and in serum [16,18]. To validate the measurement, the HSP27 concentrations in serum were measured by this approach and in a conventional laboratory immunoassay system and were compared. The HSP27 concentration detected by our assay and ELISA showed a statistical correlation. However, the HSP27 concentration measured by ELISA was significantly higher than that measured via LC–MS/MS coupled with the immunoaffinity enrichment assay. The observed difference was probably due to false positives caused by HRP enzyme reaction conditions and the possibility of cross-reactivity with other targets, which could lead to an overestimation of the target analytes [38]. Moreover, a similar phenomenon was also observed for the values obtained from the LC–MS/MS approach vs. ELISA in previous studies [39,40].

Since its discovery in 1981, CA125 still remains the gold standard serum biomarker for ovarian cancer and is widely applied to distinguish benign ovarian tumors from EOC and for monitoring the clinical course of patients with EOC [41]. However, CA125 is not approved for ovarian cancer screening or for the detection of early disease on its own because CA125 increases in nearly 50% of stage I/II EOC patients [42]. It has been widely accepted that diagnosis based on a single biomarker may not provide sufficient accuracy. Therefore, the use of multiple biomarker tests has become increasingly more common, and the corresponding multiple measurements are combined into one single score to help doctors make a better diagnostic judgment. Currently, recognized novel biomarkers, such as human epididymis protein 4 (HE4) [9,43] and candidate biomarkers including transthyretin [9], ferritin [44], metabolites [45], miRNAs [46] and so on, combined with CA125 could provide a higher diagnostic accuracy to some extent for EOC screening than that of CA125 alone. Our results also showed an HSP27 concentration that was elevated in EOC patient serum, suggesting that HSP27 could serve as an EOC biomarker to complement the standard CA125 test.

Overall, the advantages of LC–MS/MS-based targeted proteomics coupled with antibody affinity enrichment for quantification of proteins have been verified elsewhere [37,38,47]. In short, this work improves upon previous study measurements of HSP27 concentrations in cells in 2 vital ways: (1) the use of a recombination protein in pooled serum as the calibration standard decreases the deviation related to immunoaffinity enrichment of HSP27 from the matrix; (2) this antibody affinity-targeted proteomics assay both takes advantage of the enrichment capacity of the antibody and avoids nonspecificity and false positives, while the exquisite specificity of the MRM measurements overcomes issues of interferences, including nonspecific binding of the antibody. However, this study has a few limitations. Patients with cancer other than ovarian cancer were excluded from the analyses. Thus, we could not assess the specificity and sensitivity of these 2 combined biomarkers among other cancer types, and a large number of cases and other types of cancers should be included in the future.

In brief, the features of antibody affinity enrichment combined with the high quantitative accuracy and reproducibility of LC–MS/MS-based targeted proteomics could be used to determine the concentrations of any protein, especially low-concentration proteins in serum, and the method is applicable for the discovery and verification of biomarkers. It has been reported that serum fingerprints containing > 2 biomarkers have prominent advantages in the diagnosis and prognosis of disease. Therefore, the development of quantitative and multiplex assays for the simultaneous detection of multiple proteins may have potential in clinical practice using the key superiority of LC–MS/MS-based targeted proteomics assays due to their multiplexing ability.

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Supplementary data

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