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Identification of glutathione S-transferase π 1 as a prognostic proteomic biomarker for multiple myeloma using proteomic profiling

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Abstract. Multiple myeloma (MM) is a B-cell hematological malignancy with monoclonal plasma cell proliferation in the bone marrow. Early diagnosis of MM remains difficult due to the lack of specific symptoms and biomarkers. In the present study, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and the ClinProt system was used to detect potential biomarkers for MM from the bone marrow samples of 30 patients and 30 healthy controls. A total of 10 of the most significantly differentiated peaks between the patients and controls were identified. When patients with MM were compared with controls, 6 peaks with m/z values of 1,779.24, 1,866.32, 2,022.36, 2,878.9, 4,417.76 and 7,155.38 were upregulated, and 4 peaks with m/z values of 1,466.54, 1,520.02, 1,546.53 and 2,991.05 were downregulated. Of these 10 peaks, 4 peaks (pk 8, 1,866.32 Da; pk 15, 2,878.90 Da; pk 17, 2,991.05 Da; and pk 3, 1,520.02 Da) were further sequenced and identified using liquid chromatography/electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). Furthermore, the expression of fibronectin 1 and glutathione S-transferase π 1 (GSTP1) were validated in patients with MM via ELISAs. Clinical data and statistical analysis indicated that GSTP1 expression was closely associated with the clinical stage of patients with MM. High GSTP1 levels were an independent risk factor for worse prognosis in patients with MM. These results demonstrate that GSTP1 may be a novel biomarker for early diagnosis, prognosis and monitoring of minimal residual disease in MM.

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Introduction

Multiple myeloma (MM) is a monoclonal plasma cell malignancy accounting for 1% of neoplastic diseases and >10% of all hematological malignancies from cancer statistics in 2016 (1). MM is characterized by CRAB features, defined as hypercalcemia, anemia, renal insufficiency and bone lesions (2). A combinatorial treatment of bortezomib and stem cell transplantation has prolonged the overall survival time of patients with MM (3). MM remains an incurable malignancy to date, and the prognosis of MM is frequently poor due to inefficient early diagnosis (4). In general, MM is characterized by multi-step stages, including an indistinguishable early stage called monoclonal gammopathy of undetermined significance (MGUS) and an intermediate stage called smoldering MM (5) Ultimately, MM progresses to symptomatic plasma neoplasms, including intramedullary multiple myeloma and extramedullary plasmacytoma (6). Early MM has no typical features, and the presence of CRAB symptoms is usually attributed to disease progression (6). To improve long-term survival time, early diagnosis and a risk stratification assessment for MM are required. MGUS and MM exhibit few differences in global gene expression profiling (7). Understanding of the proteome of MM is essential for a better understanding of the biology of MM, and may lead to the development of more effective treatment strategies. Following diagnosis, monitoring treatment response is equally important. Despite receiving early advanced medical treatment, some patients still suffer from primary disease relapse and drug resistance (8). Traditional measurements of levels of monoclonal protein secreted by plasma cells and bone marrow (BM) have limitations (9). Thus, the International Multiple Myeloma Working Group (IMWG) revised the response criteria for diagnosis of MM to include sequencing and flow cytometry-based approaches as evaluation methods for minimal residual disease (MRD) (10). To summarize, early detection of MM combined with monitoring of MRD may improve disease treatment.

The clinical manifestations of MM are highly variable. The diagnostic criteria for MM were first established by Durie and Salmon (DS) in 1975. The DS staging system uses levels of hemoglobin, serum calcium, creatinine and the concentration of monoclonal serum protein to distinguish

patients with different prognoses (11). To eliminate the drawbacks of the traditional staging system, a new and powerful classification system based on serum β2-microglobulin and albumin levels was defined in 2005 by the IMWG (12). With in-depth research into B-cell development and plasma cell biology, MM was defined to be a heterogeneous disease accompanied by genetic alterations that are the driving events for tumor genesis (13). Further studies are being conducted to determine the initiator of clonal evolution and reveal the mechanisms involved in the process. The association between multiple myeloma cells and the BM microenvironment is also being investigated. The BM microenvironment consists of numerous stromal cells, mesenchymal stem cells, cytokines, growth factors and chemokines, which are crucial for tumor cell growth, infiltration, migration and drug resistance (14). Studies conducted on BM serum may elucidate the interaction of MM and stromal cells.

In the last 2 decades, high-throughput matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been one of the crucial, yet relatively simple proteomics tools for cancer investigation and validation across various tissues and blood serum/plasma samples (15). Some of the markers such as prostate-specific membrane antigen (PSMA) and osteopontin were associated with cancer stage and prognosis and lack of early detection (16). Therefore, the identification of novel and specific biomarkers for diagnosis and evaluation of prognosis is urgently needed. In the present study, MALDI-TOF MS based on a magnetic bead purification approach was used to detect potential biomarkers from BM samples for the first time. We supposed to find potential biomarkers for early diagnosis and a valuable therapeutic target for MM.

Materials and methods

Samples and patients. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). All participants in the study provided written informed consent. BM and serum specimens were obtained from 30 patients with MM and 30 healthy controls diagnosed at the First Affiliated Hospital of Xi'an Jiaotong University between April, 2011 and November, 2012. Patients with MM were newly-diagnosed based on the diagnostic criteria from the IMWG guidelines (17). Disease stage was defined according to the International Staging System (ISS) and DS Staging System (10,11). The clinical parameters of the patients with MM are presented in Table I. The control group consisted of 30 individuals who were suspected to have a hematological disease and were later revealed to not have hemato-oncological disease. No patients had a history of hematological neoplasms. In all cases, 3 ml BM samples were collected in BD vacutainers without anticoagulants. Next, the samples were centrifuged within 1 h of collection at room temperature at 800 x g for 10 min. The supernatant was separated and centrifuged at 4˚C at 3,500 x g for 10 min. BM samples were stored at -80˚C for subsequent analysis.

Peptidome purification and MALDI-TOF peptide profiling. The samples obtained from patients and controls were purified using the manufacturer's standard protocol. First, 10 μl of each sample was incubated with 10 μl of magnetic beads from a weak cation-exchange chromatography kit (MB-WCX Profiling kit; Bruker Corporation) at room temperature for 5 min. Next, the mixtures were loaded on the magnetic bead separator for 1 min at room temperature. The supernatant was removed from the separator and washed with washing buffer three times. Finally, 1 μl of eluted sample was manually deposited onto the MALDI AnchorChip target surface (Bruker Corporation) and overlaid with a 1 μl matrix containing 3 mg/ml α-cyano-4-hydroxy-cinnamic acid, 50% acetonitrile and 2% trifluoroacetic acid.

The mixed targets were analyzed using Autoflex III MALDI-TOF MS instrument (Bruker Corporation) with a mass range of 700-10,000 Da according to the manufacturer's measuring protocol. Mass spectra data were collected and analyzed by Flex Analysis v3.0 and ClinProTools version 2.2 (Bruker Corporation) using a standard data preparation workflow. Mass spectra calibration was performed using standard peptides. All measurements of patient and control group samples were performed in random order. According to the P-value of Wilcoxon test and Average peak intensity of different groups (Ave1 and Ave2), the top 50% most differentially expressed peaks were selected for further investigation.

Identification of peptides. Peptide sequencing and identification were performed with a Liquid chromatography/electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) system. The LC-ESI-MS/MS system contained an Acquity Ultra Performance Liquid Chromatography system (Waters Corporation) and LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Inc.). The samples were diluted in gradient elution containing formic acid and acetonitrile and then injected into a C18 trap column (Nano Acquity™ Column; Waters Corporation) for 3 min at 35˚C. The flow rate was set to 15 μl/min. Ultimately, peptides were analyzed through a C18 analytical column (Nano Acquity™ Column) for 60 min with a rate of 400 nl/min at 35˚C. The electrospray source was performed in a dynamic exclusion and data-dependent model. The full scan of MS spectra was operated with a scanning range from m/z 400-2,000. The optimal MS operating parameters were set as follows: Ionization mode: Electrospray ionization, dryer temperature: 350˚C, nebulizer pressure: 0.24 MPa (35.0 psi), flow rate: 12 l/min, Spray voltage of 1.8 kV, and scanning time of 60 min. The first scan (MS) used Orbitrap with a resolution of 100,000. The second scan (MS/MS) and character identifier used LTQ-Orbitrap. The single isotope composed of the 10 most intense ions was selected as the parent ion for MS. The acquired data were analyzed and retrieved using BioWorks Browser (BioWorks Browser 3.3.1 SP1 for Sequest™) from the International Protein Index database. The MS search criteria were set as a parent ion error of 100 ppm, a fragment ion error of 1 Da, and in non-digested mode with variable modifications at methionine oxidation

ELISA determination of serum proteins. Concentrations of GSTP1, FN1, complement 3f (C3f) and α-fetoprotein (AFP) were assayed using ELISA kits (nos. MBS700126, KE00039, 108823 and 193765, respectively) (R&D Systems, Inc.) using serum samples from 30 MM patients and 30 healthy controls.

Table I. Characteristics of patients with multiple myeloma.

Characteristics	Values
Total, n	30
Sex	
Male	17
Female	13
Age, years, mean (range)	59.6 (38-71)
DS stage, n	
I	5
II	10
III	15
ISS stage, n	
I	5
II	14
III	11
M component, n	
IgG	15
IgD	2
IgA	6
κ	3
λ	3
Nonsecretory	1
Serum M protein, g/l, mean (range)	21.0 (7-44)
Bone marrow plasma cell, %, mean (range)	33.5 (17-79)

DS stage, Durie and Salmon Staging System; ISS, International Staging System.

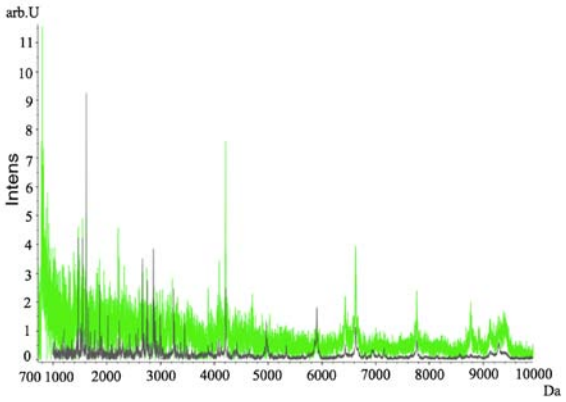

Figure 1. Bone marrow peptide fingerprints of multiple myeloma group (green) and control group (gray) with mass spectra of 700-10,000 Da.

Procedures were performed according to the manufacturer's instructions.

Statistical analysis. SPSS 25.0 (IBM Corp.) was used for all statistical analyses. Comparisons between various groups were performed by one-way ANOVA and a Student-Newman-Keuls test for post hoc comparisons. Receiver operating characteristic curve analysis was performed to determine the diagnostic capability of GSTP1 and FN1. The associations between GSPT1 level and clinical characteristics were examined by χ^2 tests. ROC analyses were performed to calculate AUCs to define threshold for GSTP1 that could be used to discriminate different groups. Comparisons of variables between high and low GTSP1 groups were analyzed by unpaired Student's t-test. Multivariate Cox models were used to determine the association between clinicopathological parameters and overall survival time. Survival analysis was conducted using the Kaplan-Meier method followed by log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Proteomic profiling of patients with MM and controls. Proteomic profiles of the MM and control groups were compared. Fractionation of BM samples from MM (gray) and control (green) groups was performed and the proteomic profiles subsequently analyzed using ClinProt-based serum peptidomics (Fig. 1). Various differentially expressed peaks within the 700-10,000 kDa mass range were detected between the 2 groups (Fig. 1). Table II summarizes the characteristics of the 10 significantly differentiated peaks. Among these 10 peaks, 6 peaks with m/z values of 1,779.24, 1,866.32, 2,022.36, 2,878.90, 4,417.76 and 7,155.38 were upregulated, and 4 peaks with m/z values of 1,466.54, 1,520.02, 1,546.53 and 2,991.05 were downregulated in patients with MM compared with healthy controls. Potential biomarkers were selected using spectral data from patients with MM and healthy controls.

Peptide identification using LC-ESI-MS/MS. Using LC-ESI-MS/MS, peptide peaks (pk 8, 1,866.32 Da; pk 15, 2,878.90 Da; pk 17, 2,991.05 Da and pk 3, 1,520.02 Da) were further identified (Fig. 2). These peptide sequences were identified as peptide fragments of C3f, GSTP1, FN1 and AFP (Table III).

Serum levels of C3f, GSTP1, FN1 and AFP in patients with MM and healthy controls. Serum concentrations of GSTP1, FN1, C3f and AFP in patients with MM and controls were measured using ELISAs. The mean concentrations of GSTP1 were 29.64±9.13 μg/l in patients with MM and 16.15±5.64 μg/l in controls. Meanwhile, the mean concentration of FN1 expression was significantly increased in the MM group (76.62±14.13 μg/l) compared with the control group (31.07±14.36 μg/l). The concentrations of GSTP1 and FN1 were significantly increased in the MM group compared with the control group ($P<0.05$; Fig. 3C and D).

GSTP1 expression is associated with clinical features of MM. The association between GSTP1 expression and the clinical features of patients with MM was investigated. A strong association was found between GSTP1 levels and the clinical stage of disease (DS stage, P=0.001 and ISS stage, P=0.018; Table IV). However, no significant differences were observed between the GSTP1 levels and age (P=0.670), sex (P=0.785) or Osteolytic bone disease (P=0.068) (Table IV). Patients were split into two groups based on ROC analysis. The cutoff value of expression of GSTP-1 is set as 19.45 μg/l.

Table II. Differentially expressed peptides between patients with multiple myeloma and healthy controls.

Index	Mass, Da	Dave	P-value	Ave1	Ave2	SD1	SD2
8	1,866.32	4.41	0.0000103	2.51	6.92	0.44	4.32
9	2,022.36	2.19	0.00134	2.3	4.49	0.29	3.43
7	1,779.24	0.98	0.0459	2.51	3.49	0.38	1.45
17	2,991.05	2.07	0.000101	4.34	4.34	2.28	0.56
32	7,155.38	0.77	0.00392	1.07	1.84	0.15	1.39
15	2,878.9	1.44	0.00998	3.28	4.72	1.44	2.08
3	1,520.02	0.74	0.0181	3.98	3.24	1.10	0.79
1	1,466.54	2.59	0.0473	7.65	5.05	4.51	2.20
4	1,546.53	1.39	0.0467	6.41	5.02	2.34	2.20
22	4,417.76	1.07	0.00382	1.46	2.53	0.31	3.56

Index, peptide peak index; mass, mass to charge ratio value; Dave, differences of average peak intensity between multiple myeloma group and healthy control group; ave1, average peak intensity of multiple myeloma group; ave2, average peak intensity of healthy control group; SD1, standard deviation of the peak intensity average of multiple myeloma group; SD2, standard deviation of the peak intensity average of healthy control group.

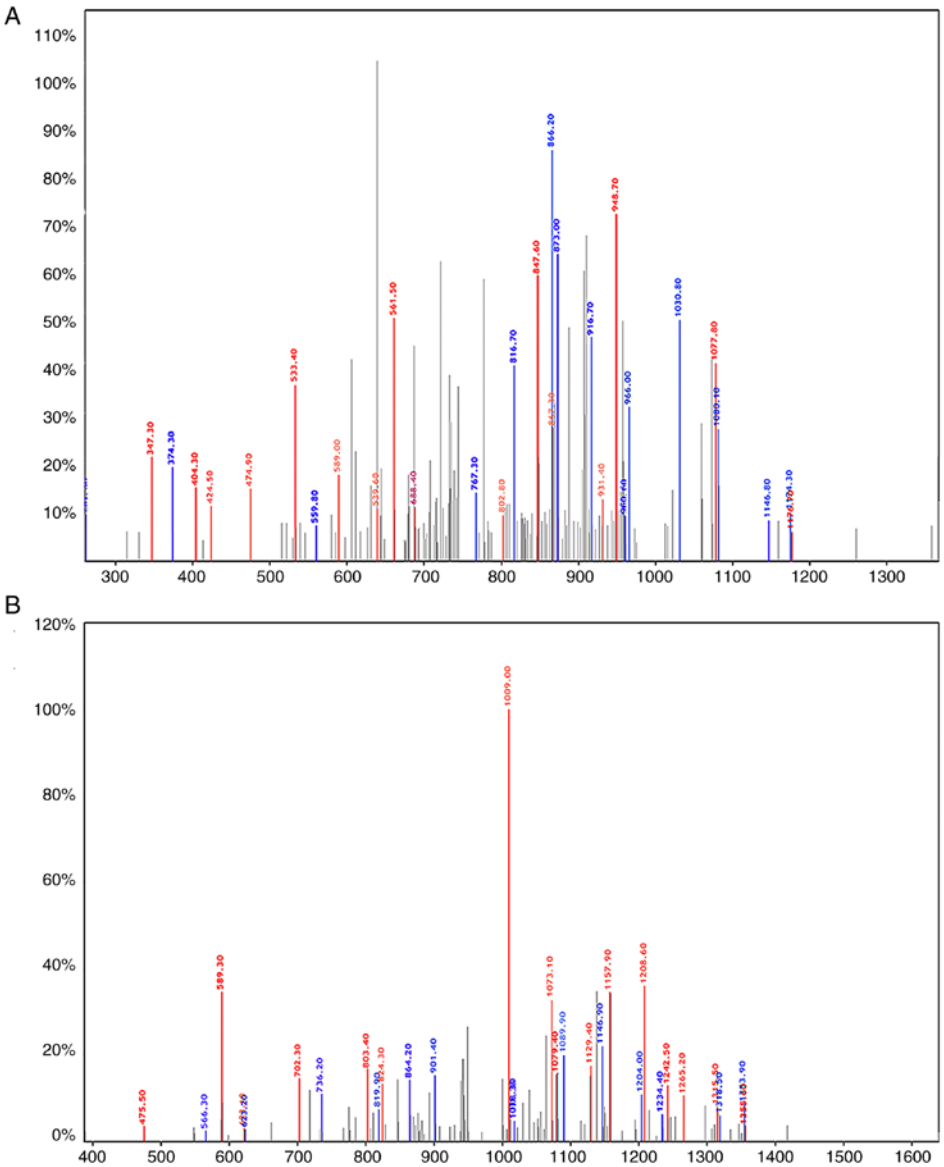

Figure 2. Liquid chromatography/electrospray ionization-tandem mass spectrometry spectrums of 4 peptides. Peaks with m/z of (A) 2,991.05 Da, (B) 2,878.9 Da.

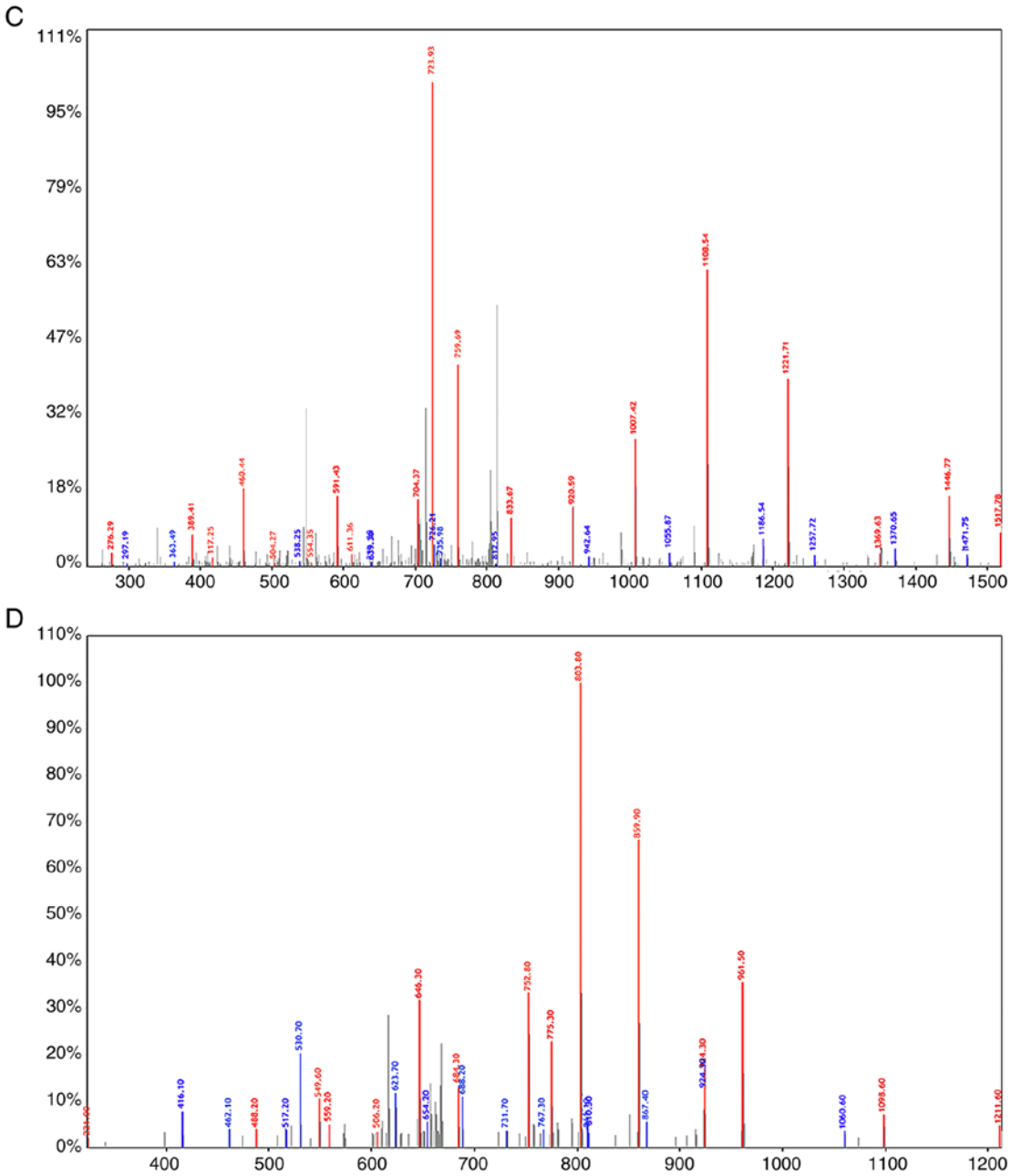

Figure 2. Continued. Liquid chromatography/electrospray ionization-tandem mass spectrometry spectrums of 4 peptides. Peaks with m/z of (C) 1,520.02 Da, and (D) 1,866.32 Da.

Student's t-test analysis revealed that the hemoglobin level and OS in the high-GSTP1 group were significantly lower than in the low-GSTP1 group (P<0.001 and P<0.05, respectively; Fig. 4B and C). Serum M protein and BM plasma cell levels were significantly higher in the high-GSTP1 group compared with the low-GSTP1 group (P<0.001 and P<0.001, respectively; Fig. 4A and D). Furthermore, multivariate Cox regression analysis indicated that the expression of GSTP1 was an independent prognostic factor for poor OS time (P=0.015; Table V). Kaplan-Meier survival analyses showed that there is significant difference between survival of high-GSTP1 and low-GSTP1 group. (P=0.018, Fig. 5A). These results indicate that GSTP1 was associated with the clinical outcome of patients with MM, and may be a valuable prognostic marker for MM.

Discussion

Similar to most human cancers, nearly all cases of MM are preceded by an asymptomatic premalignant stage such as MGUS or smoldering myeloma (2). Nearly 1% of patients with MGUS consistently progress to MM per year (18). Early detection and diagnosis of MM could significantly decrease mortality from the disease. The majority of current biomarker studies have focused on gene or protein expression in serum from patients with MM (19,20). However, these markers cannot indicate the BM microenvironment, especially for patients at early stages of MM (19). In recent years, proteomic techniques have been widely used to find potential biomarkers in various solid tumors and hematological diseases (21).

Table III. Sequence identification of selected biomarkers for patients with multiple myeloma.

Mass, Da	Peptide sequence	Identity
1,866.32	Complement 3f	R.SSKITHRIHWESASLL.R
2,991.05	Glutathione S-transferases P1	R.MLLADQGQSWKEEVVTVETWQEGSLK.A
2,878.90	Fibronectin 1	R.SYTITGLQPGTDYKIYLYTLNDNAR.S
1,520.02	α-fetoprotein	K.APQLTSSELMAITR.K

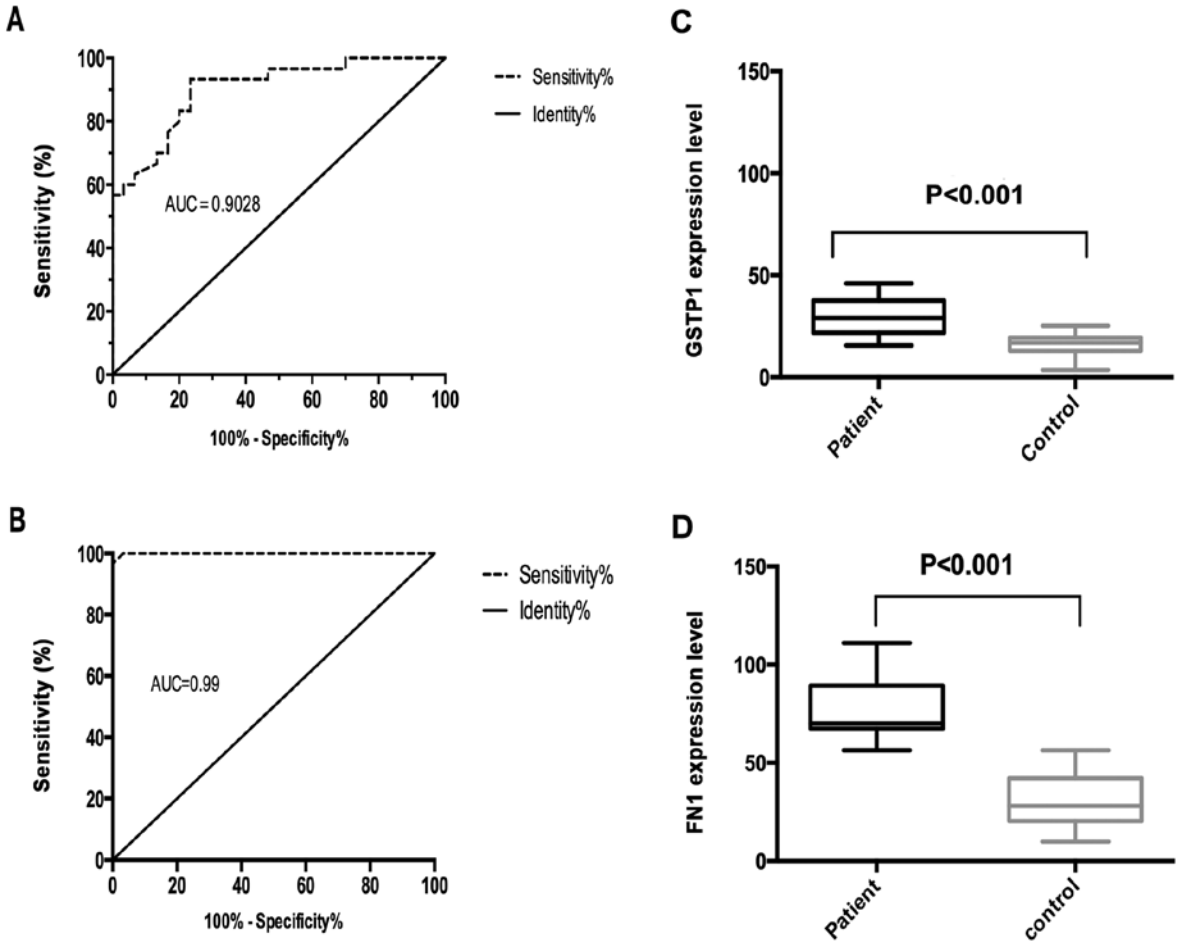

Figure 3. Expression of GSTP1 and FN1 in patients with multiple myeloma and controls detected by ELISA. Receiver operating characteristic curves with AUC values of (A) GSTP1 and (B) FN1 expression. Expression levels (μg/ml) of (C) GSTP1 and (D) FN1 in patient and control groups. FN1, fibronectin; GSTP1, glutathione s-transferase π 1.

In the present study, the BM proteomic profiles of patients with MM were established using an MB-WCX-based MALDI-TOF MS technique combined with ClinProTools. The 10 most differentially-expressed potential biomarkers between the patients and controls were analyzed. Among these biomarkers, four were down-regulated (peptides with m/z values of 1,466.54, 1,520.02, 1,546.53 and 2,991.05) and the rest were upregulated (peptides with m/z values of 1,779.24, 1,866.32, 2,022.36, 2,878.9, 4,417.76 and 7,155.38) in the MM group. Furthermore, four peptide peaks that were different between patients with MM and healthy controls were successfully identified as C3f, FN1, GSTP1 and AFP.

The complement cascade is an essential component of the human innate immune system and is an important mechanism for the detection and clearance of potential pathogens (22). C3f is an essential component of the complement cascade (23). In the alternative pathway of the complement cascade, C3b is activated by C3, which can be inactivated by factor I in the presence of one of several cofactor molecules, including factor H, complement receptor 1 and membrane co-factor protein (24). C3f is formed by the cleavage of C3b (25). The presence of C3f is associated with a predisposition for developing renal diseases, metabolic syndrome and rheumatological diseases (26,27). In addition, it has been demonstrated that C3f has a significant association with an increased risk of marginal zone lymphoma (28). To date, there have been few reports focused on the role of C3f in the pathogenesis and progression of MM. In the present study,

Table IV. Association of GSTP1 expression and clinical outcomes of patients with multiple myeloma.

Characteristics	Total	High GSTP1	Low GSTP1	P-value
Patients, n	30	13	17	
Sex, n	30			0.785
Male		7	10	
Female		6	7	
Age in years, mean ± SD	59.6±8.30	57.54±7.48	61.18±8.72	0.67
DS stage, n				0.001
I	5	0	5	
II	10	1	9	
III	15	12	3	
ISS stage, n				0.018
I	5	0	5	
II	14	5	9	
III	11	8	3	
Osteolytic bone disease	20	11	9	0.068

GSTP1, glutathione s-transferase π 1; SD, standard deviation; DS, Durie and Salmon Staging System; ISS, International Staging System.

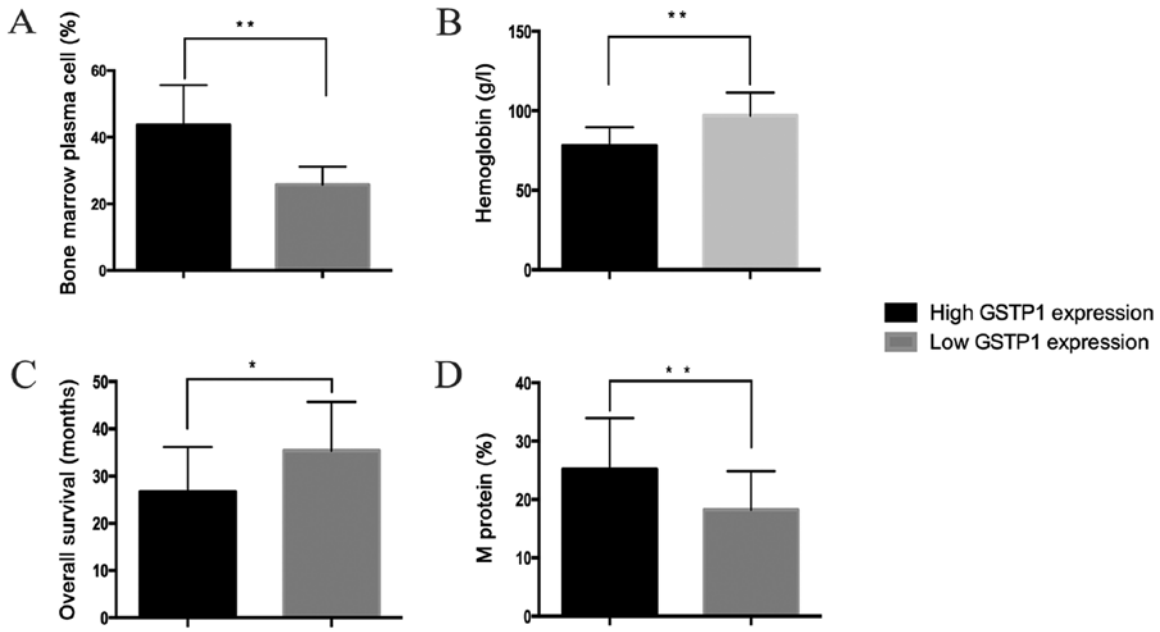

Figure 4. Comparison of clinical features in high and low GSTP1 expression groups. (A) Bone marrow plasma cell percentage, (B) hemoglobin, (C) overall survival time, (D) M protein levels. *P<0.05 and **P<0.01. GSTP1, glutathione s-transferase π 1.

the C3f level in BM samples from patients with MM was significantly increased compared with those from healthy control patients. It can be speculated that C3f participates in the genesis and development of MM. However, the association between C3f and MM pathogenesis requires further investigation.

FN1 is a member of the glycoprotein family, which is implicated in cell migration, cytoskeletal organization and oncogenic transformation, and is widely expressed in different cell lines (29,30). It has been reported that FN1 is upregulated in several tumors such as nasopharyngeal carcinoma (31) and melanoma (32). Upregulation of FN1 indicates poor prognosis in thyroid cancer and nasopharyngeal cancer (33,34). A previous study demonstrated that FN1 may be a potential biomarker for radiotherapy and drug resistance in head and neck squamous cell carcinoma (35). The exact mechanism by which FN1 induces these poor outcomes has not been investigated. High expression of FN1 suppressed apoptosis through the NF-κB pathway and was associated with migration in nasopharyngeal tumors (31). FN1 can also downregulate p53 and inhibit apoptosis in colorectal cancer (36). Since FN1 is associated with disease

Table V. Multivariate analysis of overall survival time in patients with multiple myeloma.

Characteristic	P-value	Hazard ratio	95% confidence interval	
			Lower	Upper
GSTP1	0.015	6.914	1.459	32.77
Sex	0.051	0.212	0.045	0.922
Age	0.283	0.962	0.895	1.033
DS stage	0.040	4.248	1.068	16.901
ISS stage	0.005	8.628	1.945	38.264
Serum M protein	0.002	1.135	1.046	1.232
Hemoglobin	0.004	0.892	0.826	0.964
Bone marrow plasma cell	0.001	1.122	1.054	1.195
Osteolytic bone disease	0.086	1.762	0.986	2.743

GSTP1, glutathione s-transferase π 1; DS, Durie and Salmon Staging System; ISS, International Staging System.

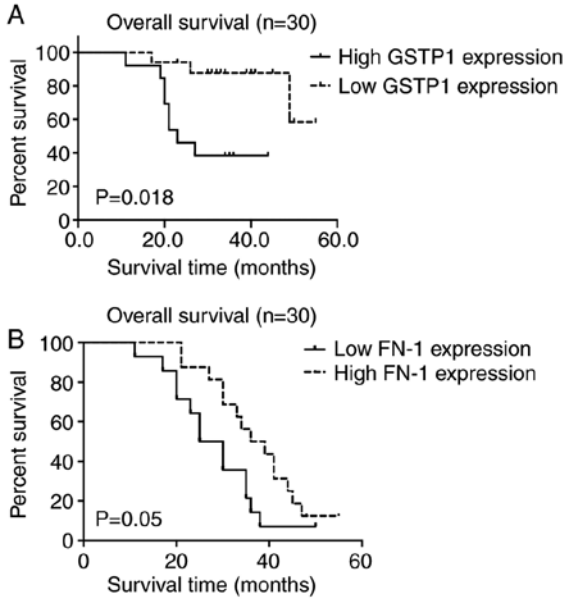

Figure 5. Effect of GSTP1 and FN1 protein expression on overall survival time in patients with multiple myeloma. (A) Survival curves for high and low GSTP1 expression. (B) Survival curves for high and low FN1 expression. FN1, fibronectin; GSTP1, glutathione s-transferase π 1.

progression and survival, it may be a prognostic biomarker in tumors. In the present study, high levels of FN1 protein expression were detected in BM samples from patients with MM. The serum level of FN1 was increased in patients with MM compared with controls. Therefore, FN1 may be a potential biomarker for MM detection.

Glutathione S-transferases (GSTs) are a superfamily of phase-II metabolic enzymes (37). The function of GSTs is essential in the antioxidant response system (38). Glutathione S-transferases π 1 is a key phase-II metabolic enzyme involved in tumorigenesis and detoxification (39). It has been reported that the expression of GSTs is increased in numerous types of human cancer and in a large number of drug-resistant tumor cell lines (40). Previous studies revealed that GSTP1 participates in tumorigenesis by regulating several kinase pathways (37,41). GST inhibitors can enhance tumor cell sensitivity to drugs leading to reversion of drug resistance (42). In addition, GSTP1 can directly influence the BM environment by stimulating aberrant redox, which causes myeloproliferative events (43). This finding is consistent with the findings of the present study, which indicate that peptides of GSTP1 were upregulated in BM samples from patients with MM compared with those from healthy control patients. Additionally, it was demonstrated that GSTP1 was upregulated in serum samples from patients with MM compared with healthy controls. Statistical analysis revealed that high levels of GSTP1 were significantly associated with the MM clinical stage and shorter OS time of patients with MM. Multivariate analysis indicated that a high level of GSTP1 may serve as an independent prognostic indicator for MM. The findings of the current study suggest that GSTP1 may not only be a biomarker of MM, but also a good indicator for monitoring MRD.

In conclusion, MB-WCX-based MALDI-TOF MS showed high sensitivity and specificity for identification of MM in patients. Peptides corresponding to GSTP1 and FN1 were confirmed by ELISA to be potential serum biomarkers in patients with MM. Furthermore, clinical data indicated that GSTP1 expression was directly associated with MM prognosis. GSTP1 may be a marker for diagnosis and prognosis of MM.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ and MZ contributed to the conception and administrative support of the study. JZ wrote the manuscript. MW attributed to provision of the study materials and collection of bone marrow samples and analysis of mass spectral data. PH and YC attributed to the collection and analysis of the experimental data. XW conducted follow-ups of the patients and analysis of clinical data. All authors have read and approved the manuscript.

Ethical approval and consent to participate

This study was approved by The Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). All participants in the study provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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