Identification of differentially expressed proteins in the gastric mucosal atypical hyperplasia tissue microenvironment

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Abstract. In the present study, the interaction of proteins in the microenvironment of gastric mucosal atypical hyperplasia was analyzed. The stromata of normal gastric mucosa (NGM) and gastric mucosal atypical hyperplasia (GMAH) tissues were purified with laser capture microdissection (LCM). The differentially expressed GMAH proteins of the NGM and GMAH tissues were identified by quantitative proteomic techniques with isotope labeling. The cross-talk between differentially expressed proteins in NGM and GMAH tissues was then analyzed by bioinformatics. There were 165 differentially expressed proteins identified from the stromata of NGM and GMAH tissues. Among them, 99 proteins were upregulated and 66 were downregulated in GMAH tissue. The present study demonstrated that these proteins in gastric mucosal atypical hyperplasia were involved in cancer-associated signaling pathways, including the p53, mitogen-activated protein kinase (MAPK), cell cycle and apoptosis signaling pathways, and were involved in cellular growth, cellular proliferation, apoptosis and the humoral immune response. The results of the present study suggest that the 165 differentially expressed proteins, including S100 calcium-binding protein A6 (S100A6) and superoxide dismutase 3 (SOD3) in the microenvironment of gastric mucosal atypical hyperplasia, are involved in the p53, MAPK, cell cycle and apoptosis signaling pathways, and serve a function in the pathogenesis of gastric cancer.

Introduction

Gastric carcinoma (GC), a serious threat to human health, is one of the most common malignancies in China, and its incidence and deaths rank first in the digestive system in 2015 (1). The occurrence of GC involves a complex pathological process associated with polygenic interactions and multi-phase evolution (2). The majority of patients experience the typical stages of normal gastric mucosa, chronic atrophic gastritis, precancerous lesions (atypical hyperplasia of gastric mucosa and intestinal metaplasia), early stages of gastric cancer, and advanced stage of disease (3). However, at present, the molecular mechanisms underlying the occurrence of GC remain unclear.

The cross-talk that exists between tumor cells and the microenvironment serves an important function in the occurrence and development of tumors (4). Tumor cells adapt to their microenvironment and exhibit corresponding biological characteristics. The tumor microenvironment refers to the internal environment in which the tumor grows, which is primarily composed of various interstitial cells, blood vessels, nerves, interstitial fluid and a small number of leucocytes (5). Tumor cells are able to induce mesenchymal cells to produce a variety of cytokines and growth factors that promote tumorigenesis and development (6). According to previous studies (7), it is possible to target the formation mechanism of the tumor microenvironment in order to prevent the proliferation and metastasis of tumor cells. Knowledge of the interaction between the microenvironment and tumor cells is expected to provide a rich theoretical basis for the treatment of tumors. The aim of the present study was to elucidate the molecular mechanisms underlying the occurrence of GC by analyzing the protein interactions in gastric mucosal atypical hyperplasia.

Materials and methods

Tissue samples. Matching specimens, including 20 cases of normal gastric mucosa (NGM) tissue and gastric mucosa atypical hyperplasia (GMAH) tissue, were collected from
The First Affiliated Hospital of University of South China between September 2016 and June 2017. The Cancer Research Institute of University of South China and The First Affiliated Hospital of University of South China are cooperative relations. Researchers from Cancer Research Institute are permitted to travel to the hospital and collect specimens with the permission of the medical ethics committee of University of South China. Specimens were collected from the stomach within 5 min of resection, and the gastric mucosal surface was washed with physiological saline prior to and following the incision. The samples were immediately frozen in liquid nitrogen and stored at -80°C. Table I presented the clinical data including tumor stage determined by the eighth edition AJCC cancer staging manual (8) of 20 patients with GC. Two senior professional pathologists from Cancer Research Institute of University of South China were asked to independently diagnose the collected tissue samples without knowing any clinical or pathological data.

Ethics statement. The human GC tissue samples were collected from The First Affiliated Hospital of University of South China according to the institutional and governmental guidelines. All patients involved in the present study provided written informed consent, and the present study was approved by the medical ethics committee of University of South China (Hengyang, China).

Preparation and staining of frozen sections. The tissue samples were removed from liquid nitrogen and placed on a cryostat device carrier (Leica Biosystems GmbH, Wetzlar, Germany). Following the addition of optimal cutting temperature compound (OCT) embedding agent (Leica Microsystems GmbH), the samples were frozen at -25°C for 20 min. Next, the samples were immobilized to the platform of the cryostat device, and frozen sections were made at a thickness of 8 µm. The frozen sections were affixed to film slides (Leica Microsystems GmbH) pretreated with ultraviolet (UV) light. Finally, the slides were fixed with 75% ethanol at 4°C for 60 sec, stained with 0.5% methyl green (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4°C for 30 sec and discolored with 95% ethanol at 4°C for 5 sec.

Laser capture microdissection (LCM). The frozen tissue sections stained with methyl green were placed on an LCM apparatus (Leica LMD6, Leica Microsystems GmbH) platform. The target tissue was outlined on the display, and the laser automatically cut the target tissue in the slice. Dissolved one tablet of protease inhibitor cocktail tablets (Roche Diagnostics, Basel, Switzerland) in 50 ml ultrapure water to prepare 5% working solutions. The tissues were collected in a tube containing 2-3 µl protease inhibitor working solutions and were frozen at -80°C for later use.

Protein extraction and isobaric tags for relative and absolute quantitation (iTRAQ) isotope labeling. The mesenchyma of the NGM and GMAH tissues were extracted using a lysis buffer (10 mM PMSF, 65 mM dithiothreitol, 7 M urea and 2 M thiourea) (GE Healthcare Life Sciences, Little Chalfont, UK) and centrifuged at 4°C, 12,000 x g for 30 min. The supernatant included the total proteins of the NGM and GMAH mesenchyma. The total proteins were extracted and quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer’s protocol. The total proteins of the NGM mesenchyma were labeled with iTRAQ reagent 114; total proteins of the GMAH mesenchyma were labeled with iTRAQ reagent 118 (both Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. A total of 100 µl ultrapure water was used to end the reaction. All protein samples were homogenized and lyophilized, and then the samples were dissolved in deionized water containing 0.1% formic acid (FA; Tedia Company, Fairfield, OH, USA). The marked samples were eluted twice with Sep-Pak C18 1 cc Vac cartridges (Waters Corporation, Milford, MA, USA) with deionized water containing 0.1% FA and then once with 50% acetonitrile (ACN) (Thermo Fisher Scientific, Inc.) containing 0.1% FA. The cleaning solution was collected and lyophilized.

Identification of differentially expressed proteins. The samples marked with iTRAQ were dissolved in 1 ml strongcation-exchange (SCX) buffer [25% (v/v) ACN and 10 mM KH2PO4, pH 2.6] for SCX separation. The two samples containing mesenchymal proteins of NGM and GMAH were mixed and loaded into a polysulfoethyl column and segregated using a 20AD high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan) with the following conditions: i) 10 mM KH2PO4 and 25% ACN, pH 2.6; ii) 10 mM KH2PO4, 350 mM KCl and 25% ACN, pH 2.6. The following settings were used: UV detection wavelength: 214/280 nm; flow rate: 200 µl/min for 60 min; salt gradient: from 5% i) at 5 min to 25% ii) at 40 min. Next, the products were concentrated by vacuum centrifugation for reverse-phase HPLC-mass spectrometry (MS) analysis. The samples were dissolved in 50 µl 5% ACN containing 0.1% FA and were loaded into a Zorbax 300SB-C18 column (Agilent Technologies, Inc., Santa Clara, CA, USA). The conditions were as follows: i) 5% ACN, 0.1% FA; ii) 95% ACN, 0.1% FA. Flow rate: 300 nl/min for 90 min. Salt gradient: from 5% i) at 5 min to 35% ii) at 70 min. The data were analyzed using QSTAR-XL (Applied Biosystems; Thermofisher Scientific, Inc.) and tandem MS (MS/MS). Finally, the IPI human database (version 3.45; URL: http://www.ebi.ac.uk/ IPI) was searched for protein information, and the confidence level was set to be >95%, and the ion peak areas of m/z 114 and 118 were integrated to perform relative quantitative analysis of proteins.

Western blot analysis. The total NGM and GMAH mesenchymal proteins were mixed with 5X loading buffer (Beyotime Institute of Biotechnology) and boiled for 5 min. The proteins had been quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Next, the samples were separated using 10% gradient SDS-PAGE gels at 30 µg per lane and transferred onto PVDF membranes (Merck KGaA). The membranes were blotted with 5% fat-free milk suspended in TBST at room temperature for 1 h, incubated at 4°C overnight with S100 calcium-binding protein A6 (S100A6) antibody (1:1,000) (sc-53950; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and superoxide dismutase 3 (SOD3) antibody (1:1,000) (sc-58427; Santa Cruz Biotechnology, Inc.), washed
and then incubated with goat anti-mouse IgG-HRP (1:2,000) (sc-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h. Detection of immunoreactivity was achieved using enhanced chemiluminescence (GE Healthcare Life Sciences).

**Immunohistochemistry.** The present study used S-P immunochemical staining kits (MXB Company, Fujian Province, China; URL: http://www.maxim.com.cn/). The NGM and GMAH tissues were fixed with 10% formalin and embedded in paraffin. The expression of S100A6 and SOD3 proteins were detected according to the manufacturer’s protocol. Briefly, 4-µm-thick sections were prepared and mounted on poly-L-lysine-coated glass slides, air-dried, deparaffinized with xylene and rehydrated in a descending ethanol series. Following microwave treatment for 20 min, endogenous peroxidase activity was suppressed using 0.3% hydrogen peroxide. The sections were treated with 5% normal goat serum (SL038) (Solarbio Life Sciences, Tongzhou Dist. Beijing, China) at room temperature for 15 min to block non-specific binding. The sections were incubated with anti-S100A6 and anti-SOD3 antibody overnight at 4°C, and then incubated with goat anti-mouse IgG-FITC (1:200) (sc-2010; Santa Cruz Biotechnology, Inc.) at room temperature for 60 min followed by horseradish peroxidase-labeled streptavidin for 5 min at room temperature. The sections were counterstained with 0.1% hematoxylin at room temperature for 30 sec. The tissue staining was observed under a light microscope at a magnification of x40. The final immunoreactive score was based on protein staining intensity and the percentage of positive cells. Staining intensity was defined as 1 (negative), 2 (yellow) and 3 (brown). The percentage of positive cells was defined as 1 (<10% positive cells), 2 (11-50% positive cells) and 3 (>50% positive cells). The final immunoreactive score was calculated as: Staining intensity x percentage of positive cells. The classification of the final score was defined as - (score 1), + (score 2-4) and +++ (score >4).

**Protein signaling pathways and interaction analysis.** Visant software (version 3.91; URL: http://visant.bu.edu) was used to analyze the interactions between proteins. Additionally, the network of direct interactions between proteins was analyzed. The Clue Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of protein signaling pathways was performed using Cytoscape software (version 2.8.2; URL: http://www.cytoscape.org). GO_BP, GO_CC and GO_MF analyses were executed with David Functional Annotation (URL: http://david.abcc.ncifcrf.gov).

**Statistical analysis.** The data are reported as the mean ± standard deviation. Statistical analysis was performed using SPSS statistical package (version 18.0; SPSS, Inc., Chicago, IL, USA) as follows: Comparison between individual subgroups was performed using the Mann-Whitney U test, and correlation analysis between groups was performed using Spearman's rank correlation test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Purified mesenchyma of NGM and GMAH tissues.** The NGM and GMAH tissues were obtained from fresh specimens of GC following surgical resection, and all tissues were confirmed by pathology. The mesenchyma of NGM and GMAH tissues were
Identification of differentially expressed proteins. The NGM and GMAH mesenchyma proteins were divided into solutions and marked using different isotopic iTRAQ. Next, the NGM and GMAH proteins were separated using a 20AD HPLC system and identified using QSTAR-XL MS/MS. A total of 165 differentially expressed proteins between the NGM and GMAH mesenchyma were identified (Table II). The G/N value (NGM/GMAH tissue) was determined as the mean protein expression level. In total, 99 proteins (G/N>1.5) were identified to be upregulated and 66 proteins (G/N<0.667) were identified to be downregulated in the GMAH mesenchyma. The expression levels of the S100A6 and SOD3 proteins were different in the mesenchyma of the NGM and GMAH tissues, and were associated with tumorigenesis in previous studies (9,10). Fig. 2 presents the MS results and the quantification of the S100A6 (Fig. 2A) and SOD3 proteins (Fig. 2B).

Discussion

The microenvironment is composed of stromal cells, immune cells and cytokines, and the tumor microenvironment has been proven to determine the biological behavior of tumor cells (11,12). It is hypothesized that the interactions of protease, cytokines and receptors in the tumor microenvironment affect the osmotic pressure and metabolism of the tumor, which may result in immune escape and neoplasia (13,14). It is important to monitor cell behavior and prevent cancer by understanding changes in the microenvironment, which serve important functions in tumor occurrence and development (4). In the present study, 165 proteins that were differentially expressed between the NGM and GMAH tissue microenvironments were screened. These proteins were demonstrated to be involved in signaling pathways associated with cancer, including the MAPK, VEGF and p53 signaling pathways, suggesting that these proteins may regulate cell growth, proliferation, apoptosis and the humoral immune response. However, the interaction network should be further characterized in follow-up studies. In the present study, the
Table II. Differentially expressed proteins between the NGM and GMAH mesenchyma.

| No. | Accession no.          | Protein name                                      | GMAH vs. NGM |
|-----|------------------------|---------------------------------------------------|--------------|
| 1   | IPI00872780.1          | ANXA4, annexin A4                                 | ↑1.5704      |
| 2   | IPI00027230.3          | HSP90B1, endoplasm precursor                      | ↑1.5704      |
| 3   | IPI00024920.1          | ATP5D, ATP synthase subunit δ                     | ↑1.5704      |
| 4   | IPI00013508.5          | ACTN1, α-actinin-1                                | ↑1.5848      |
| 5   | IPI000216135.1         | TPM1, isoform 3 of tropomyosin α-1 chain          | ↑1.5995      |
| 6   | IPI00788802.1          | TKT, transketolase variant                        | ↑1.6292      |
| 7   | IPI00647915.1          | TGLN2, 24 kDa protein                             | ↑1.6292      |
| 8   | IPI00025874.2          | RPN1                                              | ↑1.6750      |
| 9   | IPI00020599.1          | CALR, calreticulin precursor                      | ↑1.7062      |
| 10  | IPI000219219.3         | LGALS1, galectin-1                               | ↑1.7379      |
| 11  | IPI00218918.5          | ANXA1, annexin A1                                 | ↑1.7379      |
| 12  | IPI00218733.6          | ACTN1, α-actinin-1                                | ↑1.7864      |
| 13  | IPI00010796.1          | P4HB, protein disulfide-isomerase precursor       | ↑1.7864      |
| 14  | IPI00414283.5          | FN1, fibronectin 1 isoform 4 preproprotein        | ↑1.8198      |
| 15  | IPI00298547.3          | PARK7, protein DJ-1                               | ↑1.8198      |
| 16  | IPI00794402.1          | ARHGDA, 28 kDa protein                            | ↑1.8365      |
| 17  | IPI00219446.5          | PEbp1, phosphatidylethanolamine-binding protein 1 | ↑1.8879      |
| 18  | IPI0055177.1           | SERPINA1                                          | ↑1.9771      |
| 19  | IPI00029623.1          | PSMA6, proteasome subunit α type-6                | ↑1.9952      |
| 20  | IPI00026314.1          | GSN, isoform 1 of gelsolin precursor              | ↑2.0137      |
| 21  | IPI00479186.5          | PKM2                                              | ↑2.0700      |
| 22  | IPI00033494.3          | MRLC2, myosin regulatory light chain              | ↑2.0700      |
| 23  | IPI00418716            | VIM, vimentin                                      | ↑2.2492      |
| 24  | IPI00169386.5          | PGK1, phosphoglycerate kinase 1                   | ↑2.2492      |
| 25  | IPI00396321.1          | LRRC59, leucine-rich repeat-containing protein 59 | ↑2.2696      |
| 26  | IPI00027947.6          | CTRL, chymotrypsin-like protease                  | ↑2.2696      |
| 27  | IPI00884105.1          | LAMP1                                             | ↑2.3337      |
| 28  | IPI00789605.1          | MYL6                                              | ↑2.3770      |
| 29  | IPI000219018.7         | GAPDH, glyceraldehyde-3-phosphate dehydrogenase  | ↑2.3770      |
| 30  | IPI00021405.3          | LMNA, isoform A of lamin-A/C                      | ↑2.3770      |
| 31  | IPI00654755.3          | HBB, hemoglobin subunit β                         | ↑2.3987      |
| 32  | IPI00024284.4          | HSPG2                                             | ↑2.4661      |
| 33  | IPI00020987.1          | PRELP, prolargin precursor                        | ↑2.4888      |
| 34  | IPI00871843.1          | TMG2, 81 kDa protein                              | ↑2.5349      |
| 35  | IPI00418169.3          | ANXA2, annexin A2 isoform 1                       | ↑2.5349      |
| 36  | IPI00291136.4          | COL6A1, collagen α-1(VI) chain                    | ↑2.5349      |
| 37  | IPI00009771.6          | LMNB2, lamin-B2                                   | ↑2.5349      |
| 38  | IPI00742225.1          | LOC646483, DNA-binding protein TAXREB107 isoform 1| ↑2.5589      |
| 39  | IPI00297084.7          | DDOST                                             | ↑2.6062      |
| 40  | IPI00216138.6          | TAGLN, transgelin                                 | ↑2.6546      |
| 41  | IPI00025252.1          | PDIA3, protein disulfide-isomerase A3             | ↑2.6788      |
| 42  | IPI00414676.6          | HSP90AB1, heat-shock protein HSP 90-β             | ↑2.8843      |
| 43  | IPI00009904.1          | PDIA4, protein disulfide-isomerase A4             | ↑2.8843      |
| 44  | IPI00382696.1          | FLNB, isoform 2 of filamin-B                      | ↑2.9104      |
| 45  | IPI00022200.2          | COL6A3, α3 type VI collagen isoform 1             | ↑2.9922      |
| 46  | IPI00479145.2          | KRT19, type I cytoskeletal 19                     | ↑3.0202      |
| 47  | IPI00792191.1          | GATM, glycine amidinotransferase                  | ↑3.0479      |
| 48  | IPI00872814.1          | Uncharacterized protein MSN (fragment)            | ↑3.1328      |
| 49  | IPI00008274.7          | CAP1, adenylate cyclase-associated protein 1      | ↑3.1328      |
| 50  | IPI00887241.1          | LOC650788, 40S ribosomal protein S28              | ↑3.2206      |
| 51  | IPI00829626.1          | IGL@ protein                                      | ↑3.2206      |
| 52  | IPI00220278.5          | MYL9, myosin regulatory light chain 2             | ↑3.2206      |
Table II. Continued.

| No. | Accession no. | Protein name | GMAH vs. NGM |
|-----|---------------|--------------|--------------|
| 53  | IPI00021766.5 | RTN4, isoform 1 of reticulon-4 | ↑3.2510 |
| 54  | IPI00871932.1 | SPTBN1, 276 kDa protein | ↑3.3422 |
| 55  | IPI00465431.7 | LGALS3, galectin-3 | ↑3.3422 |
| 56  | IPI00333541.6 | FLNA, filamin-A | ↑3.4674 |
| 57  | IPI00221226.7 | ANXA6, annexin A6 | ↑3.5323 |
| 58  | IPI00025465.1 | OGN, mimecan precursor | ↑3.5323 |
| 59  | IPI00013296.3 | RPS18 | ↑3.5651 |
| 60  | IPI00299145.9 | KRT6C, type II cytosomelel 6C | ↑3.7665 |
| 61  | IPI00515087.2 | CTRB2, chymotrypsinogen B2 | ↑4.0933 |
| 62  | IPI00450768.7 | KRT17, type I cytosomelel 17 | ↑4.0933 |
| 63  | IPI00745872.2 | ALB, isoform 1 of albumin precursor | ↑4.2070 |
| 64  | IPI00218914.5 | ALDH1A1, retinal dehydrogenase 1 | ↑4.8309 |
| 65  | IPI00000874.1 | PRDX2, peroxiredoxin-2 | ↑4.8309 |
| 66  | IPI00000874.1 | PRDX1, peroxiredoxin-1 | ↑4.8309 |
| 67  | IPI00744153.2 | Uncharacterized protein GCG | ↑5.1520 |
| 68  | IPI00220271.3 | LOC654188, peptidylprolyl isomerase A-like | ↑5.1520 |
| 69  | IPI00883857.1 | HNRNPU | ↑5.4945 |
| 70  | IPI00020986.2 | LUM, lumican precursor | ↑5.8617 |
| 71  | IPI00010471.5 | LCP1, plastin-2 | ↑5.9172 |
| 72  | IPI00028030.3 | COMP, cartilage oligomeric matrix protein | ↑6.1958 |
| 73  | IPI00220271.3 | AKR1A1, alcohol dehydrogenase | ↑6.6050 |
| 74  | IPI00000690.1 | AIFM1, isoform 1 of apoptosis-inducing factor 1 | ↑6.6050 |
| 75  | IPI00296099.6 | THBS1, thrombospondin-1 precursor | ↑6.7935 |
| 76  | IPI00798430.1 | TF, transferrin variant | ↑7.0472 |
| 77  | IPI00410241.2 | POSTN, periostin, osteoblast specific factor | ↑7.1214 |
| 78  | IPI00646304.4 | PPIB, peptidylprolyl isomerase B precursor | ↑7.3801 |
| 79  | IPI00022391.1 | APCS, serum amyloid P-component precursor | ↑7.3801 |
| 80  | IPI00021263.3 | YWHAZ, 14-3-3 protein ζ/δ | ↑7.3801 |
| 81  | IPI00607708.3 | LDHA, isoform 2 of L-lactate dehydrogenase A chain | ↑8.3963 |
| 82  | IPI00749250.2 | ACTR2 45 kDa protein | ↑8.7108 |
| 83  | IPI00004457.3 | AOC3, membrane copper amine oxidase | ↑10.2775 |
| 84  | IPI00027463.1 | PS100A6, protein PS100 A6 | ↑10.3734 |
| 85  | IPI00215719.6 | RPL18, 60S ribosomal protein L18 | ↑10.7643 |
| 86  | IPI00014361.1 | TSTA3, GDP-L-fucose synthetase | ↑11.3766 |
| 87  | IPI00012750.3 | THBS1, thrombospondin-1 precursor | ↑12.2399 |
| 88  | IPI00010414.4 | PDLIM1, PDZ and LIM domain protein 1 | ↑12.2399 |
| 89  | IPI00744375.1 | HLA-C | ↑12.7065 |
| 90  | IPI00399007.5 | IGHG2 | ↑13.5501 |
| 91  | IPI00291006.1 | MDH2 | ↑14.4509 |
| 92  | IPI000021263.3 | Uncharacterized protein GCG | ↑15.8072 |
| 93  | IPI00020986.2 | LUM, lumican precursor | ↑15.8617 |
| 94  | IPI00798430.1 | TF, transferrin variant | ↑15.9172 |
| 95  | IPI00028030.3 | COMP, cartilage oligomeric matrix protein | ↑16.1958 |
| 96  | IPI00220271.3 | AKR1A1, alcohol dehydrogenase | ↑16.6050 |
| 97  | IPI00000690.1 | AIFM1, isoform 1 of apoptosis-inducing factor 1 | ↑16.6050 |
| 98  | IPI00296099.6 | THBS1, thrombospondin-1 precursor | ↑16.7935 |
| 99  | IPI00010414.4 | PDLIM1, PDZ and LIM domain protein 1 | ↑17.2399 |
| 100 | IPI00744375.1 | HLA-C | ↑17.7065 |
| 101 | IPI00399007.5 | IGHG2 | ↑18.5501 |
| 102 | IPI00021263.3 | Uncharacterized protein GCG | ↑19.8072 |
| 103 | IPI00025476.1 | AMY1B, pancreatic α-amylase precursor | ↑20.1086 |
| No. | Accession no. | Protein name | GMAH vs. NGM |
|-----|--------------|--------------|--------------|
| 105 | IPI00473011.3 | HBD, hemoglobin subunit δ | ↓0.1127 |
| 106 | IPI00847342.1 | KRT7, keratin 7 | ↓0.1148 |
| 107 | IPI00877792.1 | FGG, 50 kDa protein | ↓0.1259 |
| 108 | IPI00815665.1 | PRSS1, PRSS1 protein | ↓0.1259 |
| 109 | IPI00011654.2 | TUBB, tubulin β chain | ↓0.1306 |
| 110 | IPI00021885.1 | FGA, isoform 1 of fibrinogen α chain precursor | ↓0.1318 |
| 111 | IPI00009634.1 | SQRDL | ↓0.1803 |
| 112 | IPI00478003.1 | A2M, α2-macroglobulin precursor | ↓0.2014 |
| 113 | IPI00867509.1 | CORO1C, coronin-1C_i3 protein | ↓0.2291 |
| 114 | IPI00642455.2 | THBS2, thrombospondin 2 | ↓0.2291 |
| 115 | IPI00000105.4 | TUBB, tubulin β chain | ↓0.2377 |
| 116 | IPI00027720.1 | PNLIP, pancreatic triacylglycerol lipase precursor | ↓0.2421 |
| 117 | IPI00140420.4 | SND1 | ↓0.2805 |
| 118 | IPI00515061.3 | HIST1H2BJ, histone H2B type 1-J | ↓0.2884 |
| 119 | IPI00410714.5 | HBA1, hemoglobin subunit α | ↓0.2911 |
| 120 | IPI00295663.1 | ELA3A, elastase-3A precursor | ↓0.2992 |
| 121 | IPI00000105.4 | TUBB, tubulin β chain | ↓0.3020 |
| 122 | IPI00003527.5 | SLC9A3R1 | ↓0.3221 |
| 123 | IPI00788782.1 | ATP1A3, Na⁺/K⁺-ATPase α3 subunit variant | ↓0.3404 |
| 124 | IPI00028908.3 | NID2, nidogen-2 precursor | ↓0.3532 |
| 125 | IPI00186290.6 | EEF2, elongation factor 2 | ↓0.3698 |
| 126 | IPI00010779.4 | TPM4, isoform 1 of tropomyosin α-4 chain | ↓0.3767 |
| 127 | IPI00873444.1 | UBC, RPS27A 79 kDa protein | ↓0.3837 |
| 128 | IPI00031522.2 | HADHA, trifunctional enzyme subunit α | ↓0.4055 |
| 129 | IPI00156689.3 | Putative uncharacterized protein DKFZp686C15213 | ↓0.4207 |
| 130 | IPI00178926.2 | VCA1, immunoglobulin J chain | ↓0.3981 |
| 131 | IPI00021827.3 | DEFA3, neutrophil defensin 3 precursor | ↓0.3981 |
| 132 | IPI00337741.4 | APEH, acylamino-acid-releasing enzyme | ↓0.4169 |
| 133 | IPI00292530.1 | ITIH1, inter-α-trypsin inhibitor heavy chain H1 | ↓0.4270 |
| 134 | IPI00305277.5 | SLC9A3R1 | ↓0.3495 |
| 135 | IPI00260513.1 | Putative uncharacterized protein DKFZp686C15213 | ↓0.4270 |
| 136 | IPI00009027.1 | REG1A, lithostathine-1-α precursor | ↓0.4270 |
| 137 | IPI00037741.4 | APEH, acylamino-acid-releasing enzyme | ↓0.4055 |
| 138 | IPI00873444.1 | UBC, RPS27A 79 kDa protein | ↓0.3837 |
| 139 | IPI00031522.2 | HADHA, trifunctional enzyme subunit α | ↓0.4207 |
| 140 | IPI00028908.3 | NID2, nidogen-2 precursor | ↓0.3532 |
| 141 | IPI00021827.3 | DEFA3, neutrophil defensin 3 precursor | ↓0.3981 |
| 142 | IPI00337741.4 | APEH, acylamino-acid-releasing enzyme | ↓0.4055 |
| 143 | IPI000292530.1 | ITIH1, inter-α-trypsin inhibitor heavy chain H1 | ↓0.4169 |
| 144 | IPI00305277.5 | SLC9A3R1 | ↓0.3495 |
| 145 | IPI00028908.3 | NID2, nidogen-2 precursor | ↓0.3532 |
| 146 | IPI00021827.3 | DEFA3, neutrophil defensin 3 precursor | ↓0.3981 |
| 147 | IPI00337741.4 | APEH, acylamino-acid-releasing enzyme | ↓0.4055 |
| 148 | IPI000292530.1 | ITIH1, inter-α-trypsin inhibitor heavy chain H1 | ↓0.4169 |
| 149 | IPI00305277.5 | SLC9A3R1 | ↓0.3495 |
| 150 | IPI00178926.2 | VCA1, immunoglobulin J chain | ↓0.3981 |
| 151 | IPI00028908.3 | NID2, nidogen-2 precursor | ↓0.3532 |
| 152 | IPI00021827.3 | DEFA3, neutrophil defensin 3 precursor | ↓0.3981 |
| 153 | IPI00337741.4 | APEH, acylamino-acid-releasing enzyme | ↓0.4055 |
| 154 | IPI000292530.1 | ITIH1, inter-α-trypsin inhibitor heavy chain H1 | ↓0.4169 |
| 155 | IPI00305277.5 | SLC9A3R1 | ↓0.3495 |
| 156 | IPI00178926.2 | VCA1, immunoglobulin J chain | ↓0.3981 |
| 157 | IPI00028908.3 | NID2, nidogen-2 precursor | ↓0.3532 |
| 158 | IPI00021827.3 | DEFA3, neutrophil defensin 3 precursor | ↓0.3981 |
| 159 | IPI00337741.4 | APEH, acylamino-acid-releasing enzyme | ↓0.4055 |
| 160 | IPI000292530.1 | ITIH1, inter-α-trypsin inhibitor heavy chain H1 | ↓0.4169 |
| 161 | IPI00305277.5 | SLC9A3R1 | ↓0.3495 |
expression of S100A6 and SOD3 was analyzed by western blotting and immunohistochemical staining techniques, and was identified to be significantly different and associated with tumorigenesis. These results were consistent with the results of quantitative proteomics in the present study.

S100A6 is a member of the S100 protein family (15). S100A6 has a number of biological functions, including participating in the degradation and ubiquitination of β-catenin, promoting apoptosis, interacting with extracellular matrix proteins, enhancing cell metabolism and skeleton depolymerization, participating in endocytosis and exocytosis, adjusting enzyme activity, inhibiting protein kinase C-mediated phosphorylation and participating in gene transcription (16,17). A number of studies have demonstrated that S100A6 is also associated with the occurrence and development of tumors and is upregulated in several tumors, including ovarian cancer, colorectal cancer, pancreatic cancer, liver cancer, malignant melanoma and osteosarcoma (18,19). According to the results of the present study, S100A6 is upregulated in the GMAH stroma. This protein may contribute to the malignant transformation of epithelial cells of gastric mucosa and promote cell invasion and metastasis. The S100A6 protein may be a potential biomarker for monitoring malignant cell transformation.

Mammalian SODs have three subtypes, namely the cytoplasmic SOD (CuZnSOD or SOD1), mitochondrial SOD (MnSOD or SOD2) and extracellular SOD (EC-SOD or SOD3) (20). SOD3 serves an important function in maintaining the oxidation balance that prevents nuclear DNA and protein oxidative damage in the extracellular matrix and nucleus (21). Previous studies have identified that the level of SOD3 was decreased in a variety of tumors, including lung, breast and thyroid cancer, and renal cell carcinoma (10,22). SOD3 is widely expressed in normal tissues; low or no expression of SOD3 causes an imbalance in the extracellular redox environment and cancer occurs more frequently in an imbalanced environment (23). Therefore, a low or no expression of SOD3 may be a risk factor for malignant cell transformation (24). The results of the present study demonstrated that SOD3 was downregulated in GMAH stroma, which resulted in DNA damage in gastric mucosa epithelial cells and GC. Therefore, the early detection of SOD3 may predict the occurrence of GC.

As the tumor microenvironment serves a critical function in GC occurrence and development, it important to identify

| Protein | n | Low (-) | Moderate (+) | High (+++) | Positive rate, % |
|---------|---|---------|--------------|------------|-----------------|
| SOD3    |   |         |              |            |                 |
| NGM     | 20| 8       | 8            | 4          | 60.00           |
| GMAH    | 20| 14      | 4            | 2          | 30.00a          |
| S100A6  |   |         |              |            |                 |
| NGM     | 20| 13      | 4            | 3          | 35.00           |
| GMAH    | 20| 7       | 11           | 2          | 65.00a          |

*aP<0.05, GMAH vs. NGM tissues. SOD3, superoxide dismutase 3; S100A6, S100 calcium-binding protein A6; NGM, normal gastric mucosa; GMAH, gastric mucosal atypical hyperplasia.
the proteins present in the GMAH microenvironment. The present study identified a total of 165 differentially expressed proteins in GMAH stroma. These data will further clarify the molecular mechanisms of GC occurrence as well as potentially serving as prognostic markers for the early detection and diagnosis of GC.

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Figure 3. Expression of S100A6 and SOD3 proteins in the mesenchyma of NGM and GMAH tissues. (A) The expression of S100A6 and SOD3 proteins in NGM and GMAH mesenchymal tissues were detected by western blot analysis. Expression levels of proteins in two tissues were determined by densitometric analysis (n=3; P<0.01 vs. NGM). (B) Immunohistochemistry revealed the expression of the S100A6 protein in NGM and GMAH mesenchyma. Original magnification, x10 (left) and x40 (right). (C) Immunohistochemistry revealed the expression of the SOD3 protein in NGM and GMAH mesenchyma. Original magnification, x10 (left) and x40 (right). S100A6, S100 calcium-binding protein A6; SOD3, superoxide dismutase 3; NGM, normal gastric mucosa; GMAH, gastric mucosal atypical hyperplasia.

Figure 4. KEGG signal pathway analysis of differentially expressed proteins analyzed using Cytoscape software. (A) The signal pathway involved in the proteins that are upregulated in GMAH compared with NGM tissues. (B) The signal pathway involved in the proteins that are downregulated in GMAH compared with NGM tissues. KEGG, Kyoto Encyclopedia of Genes and Genomes; NGM, normal gastric mucosa; GMAH, gastric mucosal atypical hyperplasia; MAPK, mitogen-activated protein kinase; RIG, retinoic acid-induced gene; HTLV, human T-lymphotropic virus; NOD, nucleotide-binding oligomerization domain.
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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Authors' contributions**

ZWZ and CYL conceived and designed the experiments. HLZ, WM and CJL performed the experiments. LH and CSL analyzed the data. HLZ and ZWZ wrote the paper. All authors read and approved the manuscript.

**Ethics approval and consent to participate**

All patients involved in this study provided written informed consent, and the present study was approved by the Medical Ethics Committee of University of South China. Written informed consent was obtained from all participants.

**Consent for publication**

All patients provided their written informed consent for the publication of their data.

**Competing interests**

The authors declare that they have no conflicts of interest.

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