Comparative Proteomics Analysis of Gastric Cancer Stem Cells

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Abstract
Cancer stem cells (CSCs) are responsible for cancer progression, metastasis, and recurrence. To date, the specific markers of CSCs remain undiscovered. The aim of this study was to identify novel biomarkers of gastric CSCs for clinical diagnosis using proteomics technology. CSC-like SP cells, OCUM-12/SP cells, OCUM-2MD3/SP cells, and their parent OCUM-12 cells and OCUM-2MD3 cells were used in this study. Protein lysates from each cell line were analyzed using QSTAR Elite Liquid Chromatography with Tandem Mass Spectrometry, coupled with isobaric tags for relative and absolute quantitation technology. Candidate proteins detected by proteomics technology were validated by immunohistochemical analysis of 300 gastric cancers. Based on the results of LC-MS/MS, eight proteins, including RBBP6, GLG1, VPS13A, DCTPP1, HSPA9, HSPAA4, ALDOA, and KRT18, were up-regulated in both OCUM-12/SP cells and OCUM-2MD3/SP cells compared to their corresponding parent cells. RT-PCR analysis indicated that the expression level of RBBP6, HSPA4, DCTPP1, HSPA9, VPS13A, ALDOA, GLG1, and KRT18 was high in OCUM-12/SP and OCUM-2MD3/SP, in contrast with the control of parent OCUM-12 and OCUM-2MD3. These proteins were significantly associated with advanced invasion depth, lymph node metastasis, distant metastasis, or advanced clinical stage. RBBP6, DCTPP1, HSPA4, and ALDOA expression in particular were significantly associated with a poor prognosis in the 300 gastric cancer patients. RBBP6 was determined to be an independent prognostic factor. The motility-stimulating ability of OCUM-12/SP cells and OCUM-2MD3/SP cells was inhibited by RBBP6 siRNA. These findings might suggest that the eight proteins, RBBP6, GLG1, VPS13A, DCTPP1, HSPA9, HSPAA4, ALDOA, and KRT18, utilizing comparative proteomics analysis, were perceived to be potential CSC markers of gastric cancer. Of the eight candidate proteins, RBBP6 was suggested to be a promising prognostic biomarker and a therapeutic target for gastric cancer.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files. Accession numbers for protein data are included as UniProt/Swiss-Prot in Table 2.

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Introduction
Cancer stem cells (CSCs) are defined as a unique subpopulation in tumors that possess the ability to initiate tumor growth and sustain self-renewal [1]. It has been proposed that they can cause the heterogeneous lineage of cancer cells that constitute the tumor as well as play an important role in the malignant progression of carcinoma, such as distant metastasis, recurrence, and chemoresistance [2–4]. CSCs were initially identified in acute myeloid leukemia [5], but have recently been reported to exist in a wide variety of cancers, including gastric cancer [6]. The identification of CSC markers may open a new therapeutic perspective on the basis of selectively targeting this small population of cells [7,8]. Recently, it has been reported that CSCs possibly do express their own unique markers, such as aldehyde dehydrogenase 1 (ALDH1) [9], CD44 [10,11], and CD133 [12]. However, many of the published markers are not unique to CSCs. Quantitative protein expression profiling allows efficient identification of accurate and reproducible differential expression values for proteins [13]. Isobaric tags for relative and absolute quantitation (iTRAQ) combined with multidimensional liquid chromatography (LC) and tandem mass spectrometry (LC-MS/MS) analysis is emerging as a powerful methodology in the search for tumor biomarkers [14]. We previously reported that the side population (SP) cells are able to self-renew and produce non-SP cells, and that cancer cells in SP fractions possess high potential for tumorigenicity, distant metastasis [3], and chemoresistance [2]. This suggests that SP cells of gastric cancer possess cancer stem cell-like properties. Therefore, the aim of this study was to detect a novel CSC marker(s) of gastric cancer by comparing the proteomes among parent cells and stem cell-like SP cells that have been known to possess a rich CSC population [15].
Gastric Cancer Stem Cell Markers by Proteomics Analysis

Materials and Methods

Cell Cultures

Two gastric cancer cell lines, OCUM-2MD3 [16] and OCUM-12 [17], were used in this study. These cell lines were derived from diffuse-type gastric cancer. The culture condition was cultivated in Dulbecco’s modified Eagle medium (DMEM; Nikken, Kyoto, Japan) with 10% heat-inactivated fetal calf serum (FCS; Life Technologies, Grand Island, NY), penicillin and streptomycin, and 0.5 mM sodium pyruvate, and incubated at 37°C. OCUM-12/SP and OCUM-2MD3/SP cell lines were SP cells that were evaluated by a flow cytometric analysis using Hoechst 33342 from their parent cell lines, OCUM-2MD3 and OCUM-12, respectively. Sorting was performed three times to establish a stable population of SP-enriched cells. After a three month incubation period post-sorting, OCUM-12/SP cells (6.5%) and OCUM-2MD3/SP cells (12.2%) still represented a high percentage of the SP fraction, compared to parent OCUM-12 (3.2%) and OCUM-2MD3 (6.3%) cells [Figure S1]. Subsequently, these SP-enriched cells with a stable population were the cell lines used for the analysis, as previously reported [18].

Human Tissue Specimens and Patient Information

Tissue specimens were obtained from 300 patients diagnosed with gastric cancer permitted operations at Osaka City University. Table 1 shows the clinicopathologic characteristics of the 300 gastric cancer patients. There were 208 male and 92 female patients, with the median age of 64 years (range, 28-85 years) at the time of operation. The diagnoses were confirmed by at least two people. Staging was determined in accordance with the Japanese classification of gastric carcinoma (14th edition) [19]. This study was approved by the Osaka City University Ethics Committee (Osaka, Japan). Written informed consent from the donor was obtained for use of this sample in research.

Protein Identification and Quantification by QSTAR Elite LC-MS/MS

The cancer cells (60 μg each) were homogenized and then lysed using either 100 μL of T-PER lysis buffer (Thermo Scientific) or 500 μL of 9 M Urea, and 2% CHAPS lysis buffer with a protease inhibitor. Subsequently, the cell lysate was then treated by ultrasonication. After acetone precipitation, protein concentrations were measured by BCA Protein Assay (Pierce, IL, USA). Reduction, alkylation, digestion, and subsequent peptide labeling of 50 μg of peptide for each sample were performed using the AB Sciex iTRAQ Reagent Multi-Plex Kit (AB Sciex, Concord, ON, Canada) [20]. The iTRAQ-labeled samples were loaded onto an ICAT cation exchange cartridge (AB Sciex). The peptides were eluted as six fractions (1 mL KCL solution of 10, 50, 70, 100, 200, and 350 mM), and the supernatant of each was evaporated within a vacuum centrifuge. Samples were then desalted and concentrat-ed using a vacuum centrifuge. Samples were then desalted and concentrat-ed using Sep-Pak Light C18 cartridges (Waters Corporation, Milford, MA), evaporated within a vacuum centrifuge, resuspended in 20 μL of 0.1% (v/v) formic acid, and subsequently applied onto QSTAR Elite LC-MS/MS. Each sample was run for 150 minutes. MS/MS data was searched as the digestion enzyme and methyl methanethiosulfonate as the cysteine modification. In order to remove redundant hits and comparative quantitation, the search results obtained were further processed by ProteinPilot software using the Paragon Algorithm. This resulted in the minimal set of justifiable identified proteins. All reported data was used with a 95% confidence cut-off limit. Relative quantitation of peptides was calculated as a ratio by dividing the iTRAQ reporter intensity. The ratios of peptides that support the existence of one protein were averaged for the relative protein quantitation. Thereafter, the ProteinPilot analysis and Ingenuity pathway analysis (IPA) (Ingenuity System, Mountain View, CA) were performed. After performing a Simple t-test on one of the calculated averaged protein ratios against 1 to assess the validity of the protein expression changes, a p-value was reported. Protein ratios with a p-value of less than 0.05 were considered reliable. It should be known that in 90% of the iTRAQ experimental runs done previously, the standard deviations of the protein ratios, which stem from technical variations, were reported to be less than 0.3. Therefore, expression changes greater than 1.2-fold or less than 0.8-fold of normalized expression levels were considered to be outside the range of technical variability. We also performed a non-labeled analysis, and detected the presence of proteins only within OCUM-12/SP cells and OCUM-2MD3/SP cells, but not within parent cells [21]. Each sample was run twice. The applied LC-MS/MS examination coupled with iTRAQ technology have been reported as a reliable quantitative method for protein expression, being even more sensitive than the western blot which depends on the type of applied antibodies [22].

IPA and Selection of Candidate Proteins

The IPA database is primarily used in the field of proprietary ontology, containing up to 300,000 biological articles including genes, proteins, molecular and cellular processes. Therefore, IPA was employed for the analysis of protein molecular functions, localization. In addition, detailed information regarding the functions and cellular locations of the identified proteins was obtained. Based on the results of LC MS/MS and IPA analyses, proteins that were observed to be over-expressed in SP cell-lines, when compared to their corresponding frequency of expression in parent cell-lines, were selected as candidate biomarkers for SP cells of gastric cancer. The identification of networks of interacting proteins, as well as functional groups and pathways was generated by IPA, and the analysis depends on the previously characterized associations.

Quantitative Real-time Reverse Transcription-polymerase Chain Reaction (RT-PCR)

Gastric cancer cells were cultured. And the total cellular RNA was extracted using RNeasy Mini Kit (QIAGEN, Carlsbad, CA). cDNA was prepared from 2 μg RNA using random primers (Invitrogen). To determine fold changes in each gene, RT-PCR was performed on the ABI Prism 7000 (Applied Biosystems, Foster City, CA), with commercially available gene expression assay expressions (Applied Biosystems) for RBBP6 (retinoblastoma binding protein 6; Hs00544663), HSPA4 (heat shock 70kDa protein 4; Hs00382894), HSPA9 (heat shock 70 kDa protein 9; Hs00269818), GLG1 (Golgiglycoprotein 1; Hs00939452), DCTPP1 (dCMP diphosphatase 1; Hs00225433), VPS13A (vacuolar protein sorting 13 homolog A; Hs00362891), CK18 (keratin 18; Hs029277403), ALDOA (aldolase A; Hs00605108), CD44 (Hs01075862), CD133 (Hs01009250) and NANO5 (Hs02387433). GAPDH (SIGMA) was used as an internal standard to normalize mRNA levels. The threshold cycle (Ct) values were used to calculate the relative expression ratios between control and treated cells.

Western blot analysis

Expression level of RBBP6 and ALDOA in cancer cells was examined as follows. Cell lysates were collected after different treatments. After the protein concentration of each sample was

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adjusted, electrophoresis was carried out using 10% Tris/Gly gels (Life Technologies, Carlsbad, CA). The protein bands obtained were transferred to an Immobilon-P Transfer membrane (Amer- sham, Aylesbury, UK). Then, the membrane was placed in PBS-T solution containing anti-RBBP6 (WH0005930M1, Sigma-aldrich, MO, USA), anti-ALDOA (HPA004177, ATLAS), and anti-β-actin (1:300 dilution; Sigma-aldrich), and allowed to react at room temperature for 2 hours. The levels of specific proteins in each lysate were detected by enhanced chemiluminescence using ECL plus (Amersham) followed by autoradiography.

Small Interfering RNA Design

The sequences for RBBP6 small interfering RNA (siRNA) are designed as follows: siRBBP6 sense, 5’-GAAAGAAGAAUAUCUGAU-tt-3’; antisense, 5’- AUCAGUAUAUCUUCUUUCg-3’, and nontargeting siRNA (negative-siRNA) was purchased from Ambion (Life Technologies, Carlsbad, CA). OCUM-12/SP and OCUM-2MD3/SP cells were prepared at 60% confluence in six-well dishes. The transfection mixture was prepared by adding 150 μL of Opti-MEM including 9 μL of Lipofectamine RNA iMAX Regant (Life Technologies) to 150 μL of Opti-MEM including 90 pmol of siRNA and incubating for 5 min at room temperature. Finally, the above transfection mixture was added to prepared six-well dish. Twenty-four hours after transfection, RT-PCR was performed.

Wound-healing Assay

Cancer cells were cultured in 96-well plates (Essen Instruments, Birmingham, UK). After the cells reached semi-confluence, a wound was created in the cell monolayer with the 96-well by WoundMaker (Essen Bioscience, MI, USA). Scratched fields were taken pictured every 3 hours and was monitored with Incucyte Live-Cell Imaging System and software (Essen Instruments). The degree of cell migrations was analyzed 24 hours after wound treatment as a percentage of wound confluence. The mean of 4 fields was calculated as the sample value.

Invasion Assay

We used the chemotaxis cell chambers (Kubota, Osaka, Japan) with a 12-μm pore membrane filter coated with 50-μg Matrigel (Collaborative Research Co., Bedford, MA). The chamber (upper component) was placed in a 24-well culture plate (lower component). Gastric cancer cells were re-suspended to a final concentration of 5×10^3 cells/mL. Next, 500-μL lower components. After incubation for 48 h, cancer cells on the upper surface of the membrane were removed by wiping and stained with hematoxylin. Cancer cells that invaded through a filter coated with Matrigel into the lower membrane were manually counted under a microscope at ×200 magnification. Six randomly chosen fields were counted for each assay. The mean of four fields was calculated as the sample value. For each group, the culture was done in triplicate.

Validation of Protein Expression by Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue samples that were deparaffinized in xylene and dehydrated through graded ethanol. The sections were heated for 10 minutes at 105°C by autoclave in Target Retrieval Solution (DAKO). The samples were subsequently incubated with 3%
hydrogen peroxide to block endogenous peroxidase activity. The following antibodies were used in the immunohistochemical process: anti-RBBP6 (retinoblastoma binding protein 6; WH0005930M1, 6.1000; Sigma-Aldrich), anti-GL1 (Golgi glycoprotein 1; HPA010815, 1:50; ATLAS), anti-VPS13A (vacuolar protein sorting 13 homolog A; NBPI-85642, 1:500; Novus Biologicals), anti-ALDOA (aldolase A, fructose-bisphosphate; HPA004177, 1:400; ATLAS), anti-DCTPP1 (dCTP pyrophosphatase 1; HPA002032, 1:200; ATLAS), anti-HSPA4 (heat shock 70 kDa protein 4; HPA000898, 1:200; ATLAS), anti-HSPA9 (heat shock 70 kDa protein 9; HPA004177, 1:400; ATLAS), and anti-KRT18 (keratin 18; ab668, 1:500; Abcam). The samples were incubated with each antibody overnight at approximately 4°C. Thereafter, samples were incubated in appropriated immunoglobulin G for 10 minutes, followed by three washes with PBS. All samples were then treated with streptavidin-peroxidase reagent, and incubated in PBS diaminobenzidine and 1% hydrogen peroxide (vol/vol), followed by counterstaining with Mayer’s hematoxylin.

Immunohistochemical Evaluation

RBBP6, GL1, VPS13A, DCTPP1, HSPA9, HSPA4, ALDOA, and KRT18 expression levels were evaluated by both intensity of staining and proportion of stained tumor cells. The staining intensity was scored on a scale of 0-3 (0 = no, 1 = mild, 2 = moderate, 3 = intense). Staining proportions were scored on a scale of 0–4 (the percentage was different with each antibody) based on the percentage of positively stained cells. Therefore, the final staining score, which was calculated as a multiple of the staining intensity score and the staining proportion score, would be on a scale of 0–12. Expression levels of DCTPP1 were considered positive when it received a score of 3. Expression levels of HSPA4 were considered positive when it received a score of 6. Expression levels of ALDOA, KRT18, VPS13A, and GL1 were considered positive when each received a score of 6. RBBP6, the evaluation of which only the staining proportion score was used for calculation, was considered positive when it received a score of 3. HSPA9, the evaluation of which only the staining intensity score was used for calculation, was considered positive when it received a score of 3. All evaluations were made by two observers who were unaware of clinical data and outcome. When a discrepant evaluation between the two independent observers was found, the slides were rechecked and reevaluated after discussion.

Statistical Analysis

The SPSS software program (SPSS Japan, Tokyo, Japan) was used for data analysis. Statistical significance of the associations between the expression of proteins and the various clinicopathological variables, including age, sex, macroscopic type, tumor differentiation, total number of resected lymph node, and type of surgery (D1 or D2 gastrectomy) was evaluated using Fisher’s and Chi-squared tests. Survival curves were calculated from the day of surgery to the time of death or to the last follow-up observation using the Kaplan-Meier Method. Additionally, any differences between survival curves were assessed using the Log-rank Test. Multivariate analyses were performed according to the Cox Regression Model to determine the associations between clinicopathological variables and mortality. P-values of <0.05 was considered statistically significant.

Results

The stemness of gastric cancer cell lines

The percentages of SP cells were higher in the OCUM-12/SP and OCUM-2MD3/SP cells than in their parent OCUM-12 and OCUM-2MD3 cells (Figure S1). Cancer stem cell markers of SP cells, OCUM-12/SP and OCUM-2MD3/SP, such as CD44, CD133, and NANOG, were analyzed by RT-PCR. The expression level of these markers was significantly increased in both SP cell lines, in comparison with their parent cell lines (Figure S2). The number of spheroid colony was significantly higher in both OCUM-12/SP and OCUM-2MD3/SP cells than their parent OCUM-12 and OCUM-2MD3 cells (data not shown).

Detection of Candidate Proteins

We investigated whether proteins were differentially or independently expressed in SP cells, and compared our findings to those of their parent cells using QSTAR Elite LC-MS/MS. In analyzing biological processes, and with a 95% confidence cut-off limit and p<0.05, we identified that proteins were indeed differentially expressed. The results of these findings are presented in Figure 1. Most of the proteins were over-expressed in the cytoplasm of tumor cells (Figure 1A). The P value included in the ingenuity analysis is stated in Table S1. These proteins were determined to be related to cellular processes, such as cell death, metabolism, cellular organization, DNA metabolism, protein degradation, and processing of RNA (Figure 1B). The top canonical pathways associated with these targets and identified by IPA are shown in Table S2.

When compared to their corresponding parent cells, 40 proteins were up-regulated in OCUM-12/SP, and 35 proteins were up-regulated in OCUM-2MD3/SP. Among these proteins, eight were up-regulated in both OCUM-12/SP and OCUM-2MD3/SP cells, whereas no such association was observed between their corresponding parent cells (Table 2 and Figure 1C). Of these eight proteins, the three proteins, RBBP6, GL1, and VPS13A, were independently detected in both SP cell lines, but not in their corresponding parent cells. The five proteins, DCTPP1, HSPA9, HSPA4, ALDOA, and KRT18, were significantly over-expressed by 1.2-fold in both SP cells when compared to their corresponding parent cells.

The mRNA expression level of these 8 candidate molecules, RBBP6, HSPA4, DCTPP1, HSPA9, VPS13A, ALDOA, GL1, and CK18 was increased in OCUM-12/SP (9.15 fold, 9.36 fold, 4.14 fold, 7.90 fold, 2.08 fold, 1.46 fold, 3.44 fold, and 1.99 fold, respectively) and OCUM-2MD3/SP (6.15 fold, 1.71 fold, 2.33 fold, 2.30 fold, 2.03 fold, 1.32 fold, 1.35 fold, and 1.31 fold, respectively) cells, in comparison with those of the control parent OCUM-12 and OCUM-2MD3 cells (Figure 1D). Western blot analysis indicated that the expression level of RBBP6 and ALDOA was high in CUM-12/SP and OCUM-2MD3/SP cells, in comparison with that OCUM-12 and OCUM-2MD3 cells (Figure S3).

The network presented in Figure 1E was generated by IPA, and the analysis depends on the previously characterized and reported protein interactions. Thus, RBBP6, which was observed to be over-expressed in CSC-like SP cells, OCUM-12/SP cells and OCUM-2MD3/SP cells, was directly related to HSPA4 and Rb, and indirectly associated with Hsp90 and TAGLN2. HSPA4, Hsp90 and TAGLN2 were also found to be up-regulated in CSC-like SP cells.
Effect of siRBBP6 transfection on the migration and invasive abilities of gastric cancer cells

Figure 2A shows that siRBBP6 transfection significantly decreased mRNA expression level of both SP cell lines (OCUM-12/SP was 12%, p<0.01, OCUM-2MD3/SP was 2.5%, p<0.01), in compared with that of negative-siRNA transfection. RBBP6 siRNA knockdown significantly decreased the invasion (Figure 2B) and migration activity (Figure 2C) of both SP cells.

Immunohistochemical Assessment of Candidate Proteins and their Association with Clinicopathological Features

RBBP6, DCTPP1, and HSPA9 were observed to be primarily expressed in the cytoplasm and nuclei of gastric cancer cells. GLG1, VPS13A, HSPA1, HSPA4, ALDOA, and KRT18 were observed to be primarily expressed in the cytoplasm (Figure 3A).

In normal epithelial cells, RBBP6, GLG1, VPS13A, DCTPP1, and HSPA9 expression were found some cells in the epithelial gland. KRT18 were expressed in most epithelial cells. HSPA4 and ALDOA expression was not found in normal cells. BBP6, DCTPP1, and HSPA9 were expressed in the cytoplasm and nuclei of normal epithelial cells. GLG1, VPS13A, HSPA9, and KRT18 were observed in the cytoplasm of epithelial cells (Figure 3B).

We explored the association between the expression level of the eight candidate proteins and the clinicopathological features. Number of cases to each score for the eight targets was shown in Table S3. These eight proteins were determined to be associated with potentially malignant processes, such as distant metastasis, lymph node (LN) metastasis, invasion depth, or stage advancement (Table 3). The calculated p-values were as follows: RBBP6 was significantly associated with invasion depth (p<0.001), LN metastasis (p<0.001), distant metastasis (p=0.013), and clinical stage (p<0.001); GLG1 was significantly associated with only distant metastasis (p=0.045); VPS13A was significantly associated with invasion depth (p=0.005), LN metastasis (p<0.001), and stage advancement (p=0.003); DCTPP1 was significantly associated with invasion depth (p<0.001), LN metastasis (p<0.001),
Table 2. Proteins increased in both OCUM-12/SP and OCUM-2MD3/SP cells compared to their parent cells detected by QSTAR Elite LC/MS/MS.

| Symbol | Protein Name                          | GI Number | UniProt/Swiss-Prot Ratio | p value | Location | Type |
|--------|--------------------------------------|-----------|--------------------------|---------|----------|------|
| RBBP6  | retinoblastoma binding protein 6      | 74762440  | Q7Z6E9                  | NA      | N        | En   |
| GLG1   | golgi glycoprotein 1                  | 218512060 | Q92896                  | NA      | C        | O    |
| VPS13A | vacuolar protein sorting 13 homolog A (S. cerevisiae) | 71152975  | Q96RL7                  | NA      | C        | Tp   |
| DCTPP1 | dCTP pyrophosphatase 1                | 74733624  | Q9H773                  | 0.0206  | C        | En   |
| HSPA9  | heat shock 70 kDa protein 9 (mortalin) | 21264428  | P38646                  | 0.0006  | C        | O    |
| HSPA4  | heat shock 70 kDa protein 4           | 206629954 | P34932                  | 0.009   | C        | O    |
| ALDOA  | aldolase A, fructose-bisphosphate     | 113606    | P00755                  | <0.0001 | C        | En   |
| KRT18  | keratin 18                           | 125083    | P05783                  | 0.0003  | C        | O    |
| NAMPT  | nicotinamide phosphoribosyltransferase| 1172027   | Q4L4Y1                  | 0.036   | C        | O    |
| ACO2   | aconitase 2, mitochondrial            | 6682675   | Q99798                  | 0.0312  | C        | En   |
| AKR1B1 | aldo-keto reductase family 1, member B1 (aldo reductase) | 113596   | P15121                  | 0.0021  | C        | En   |
| AKR1B10| aldo-keto reductase family 1, member B10 (aldo reductase) | 20531983 | Q83905                  | 0.046   | C        | En   |
| CBR1   | carbonyl reductase 1                  | 118519    | P16152                  | 0.0456  | C        | En   |
| GAPDH  | glyceraldehyde-3-phosphate dehydrogenase | 120649   | P04406                  | 0.0105  | C        | En   |
| GART   | phosphoribosylglycinamid-formyltransferase, phosphoribosylglycinamid synthetase, phosphoribosylaminoimidazole synthetase | 131616   | P21002                  | 0.036   | C        | En   |
| GSTM3  | glutathione S-transferase mu 3 (brain) | 21264423 | P21266                  | 0.0018  | C        | En   |
| HSP90AA1| heat shock protein 90 kDa alpha (cytosolic), class A member 1 | 92090606 | P07900                  | 0.0022  | C        | En   |
| HSP90AB1| heat shock protein 90 kDa alpha (cytosolic), class B member 1 | 17865718 | P08238                  | 0.0068  | C        | En   |
| HSPD1  | heat shock 60 kDa protein 1 (chaperonin) | 129379    | P10809                  | 0.056   | C        | En   |
| MTHFD1 | methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1, methylenetetrahydrofolate cyclohydrolase, formylenetetrahydrofolate synthetase | 115206   | P11586                  | 0.0196  | C        | En   |
| PRDX6  | peroxiredoxin 6                       | 1718024   | P30041                  | 0.015   | C        | En   |
| TALDO1 | transaldolase 1                       | 6640892   | P37837                  | 0.0044  | C        | En   |
| UGDH   | UDP-glucose 6-dehydrogenase           | 6175086   | B1YLD1                  | 0.0318  | N        | En   |
| PFKP   | phosphofructokinase, platelet         | 1346355   | Q01813                  | 0.0019  | C        | K    |
| PKM2   | pyruvate kinase, muscle               | 20178296  | P14618                  | 0.027   | C        | K    |
| ACTN4  | actinin, alpha 4                      | 13123943  | I4AQ0Q                  | 0.0011  | C        | O    |
| CANX   | calnexin                              | 543920    | P27824                  | 0.0005  | C        | O    |
| HSPA2  | heat shock 70 kDa protein 2           | 1708307   | P54652                  | 0.0358  | C        | O    |
| RPL6   | ribosomal protein L6                  | 1350762   | Q02878                  | 0.0027  | C        | O    |
| TAGLN2 | transgelin                            | 586000    | P37802                  | 0.0134  | C        | O    |
| WDR1   | WD repeat domain 1                    | 12643636  | G2TF25                  | 0.0381  | ES       | O    |
| PTMA   | prothymosin, alpha                    | 135834    | P06454                  | 0.0401  | N        | O    |
| Symbol     | Protein Name                              | GI Number | UniProt/Swiss-Prot | Ratio*  | p value | Location† | Type‡ |
|------------|-------------------------------------------|-----------|--------------------|---------|---------|-----------|-------|
| PPP2R1A    | protein phosphatase 2, regulatory subunit A, alpha | 143811355 | P30153             | 1.439   | 0.0003  | C         | phosphatase |
| SQSTM1     | sequestosome 1                             | 74735628  | Q13501             | 1.877   | 0.0038  | C         | TR    |
| EIF3B      | eukaryotic translation initiation factor 3, subunit B | 218512094 | P55884             | 1.234   | 0.0229  | C         | TR    |
| RPSA       | ribosomal protein SA                       | 125969    | P08685             | 1.31    | 0.0017  | C         | TR    |
| ATP2A1     | ATPase, Ca2+ transporting, cardiac muscle, fast twitch 1 | 12643544  | G27F52             | 1.058   | 0.0305  | C         | Tp    |
| ATP5B      | ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide | 11454-49 | P06576             | 1.594   | 0.0111  | C         | Tp    |
| ETF4A      | electron-transfer-flavoprotein, alpha polypeptide | 119636    | P13804             | 1.473   | 0.0003  | C         | Tp    |
| KPNB1      | karyopherin (importin) beta 1             | 20981701  | Q14974             | 1.188   | 0.0397  | N         | Tp    |
| OCUM-2MD3/SP | OCUM-2MD3/5P ratio                     |           |                    |         |         |           |       |
| RBBP6      | retinoblastoma binding protein 6          | 74762440  | Q7Z6E9             | †        | NA      | N         | En    |
| GLG1       | golgi glycoprotein 1                      | 218512060 | Q92896             | †        | NA      | C         | O     |
| VPS13A     | vacuolar protein sorting 13 homolog A (S. cerevisiae) | 71152975  | Q96RL7             | †        | NA      | C         | Tp    |
| DCTP1      | dCTP pyrophosphatase 1                    | 7473624   | Q9H773             | 1.467   | 0       | C         | En    |
| HSPA9      | heat shock 70 kDa protein 9 (mortalin)    | 21264426  | P38646             | 1.778   | 0.0005  | C         | O     |
| HSPA4      | heat shock 70 kDa protein 4               | 206729934 | P34932             | 1.27    | 0.01    | C         | O     |
| ALDOA      | aldolase A, fructose-bisphosphate         | 113606    | P00759             | 1.533   | 0.035   | C         | En    |
| KRT18      | keratin 18                                | 125083    | P05783             | 1.618   | 0.0066  | C         | O     |
| MDH2       | malate dehydrogenase 2, NAD (mitochondrial) | 215274114 | P40916             | 1.604   | 0.0244  | C         | En    |
| PROX1      | peroxiredoxin 1                          | 548453    | Q06830             | 1.334   | 0.0003  | C         | En    |
| DLST       | dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex) | 206729909 | P36957             | 1.277   | 0.0483  | C         | En    |
| PDI4A      | protein disulfide isomerase family A, member 4 | 119530    | P13667             | 1.494   | 0.0353  | C         | En    |
| PYG8       | phosphorylase, glycogen; brain            | 20178317  | P11216             | 1.87    | 0.0071  | C         | En    |
| NARS       | asparaginyl-tRNA synthetase               | 3915059   | O53857             | 2.239   | 0.0089  | C         | En    |
| UBA1       | ubiquitin-like modifier activating enzyme 1 | 24418865  | P23314             | 1.353   | 0.0467  | C         | En    |
| P4H4B      | prolyl 4-hydroxylase, beta polypeptide    | 2507460   | P07237             | 1.852   | 0.043   | C         | En    |
| LDHA       | lactate dehydrogenase A                   | 126047    | P00338             | 1.373   | 0.0014  | C         | En    |
| PP1B       | peptidylprolyl isomerase B (cyclophilin B) | 215273869 | P23284             | 1.783   | 0.0112  | C         | En    |
| GPI        | glucose-6-phosphate isomerase             | 17380385  | P06744             | 2.023   | 0.0027  | ES        | En    |
| SSB        | Spgner syndrome antigen 8 (autoantigen La) | 125985    | P05455             | 1.591   | 0.0222  | N         | En    |
| HDGF       | hepatoma-derived growth factor            | 1708157   | P51858             | 2.537   | 0.0468  | ES        | growth factor |
| HYOU1      | hypoxia up-regulated 1                    | 10720185  | QY4L1              | 1.643   | 0.0005  | C         | O     |
| HSP90B1    | heat shock protein 90 kDa beta (Grp94), member 1 | 119500    | P14625             | 3.233   | 0.0003  | C         | O     |
| AGR2       | anterior gradient 2 homolog (Xenopus laevis) | 67462105  | O95994             | 2.172   | 0.0107  | ES        | O     |
| Symbol       | Protein Name                                                                 | GI Number | UniProt/Swiss-Prot | Ratio | p value | Location | Type       |
|--------------|------------------------------------------------------------------------------|-----------|--------------------|-------|---------|----------|------------|
| HIST1H1E     | histone cluster 1, H1e                                                       | 121919    | P10412             | 1.562 | 0.0353  | N        | O          |
| HNRNPA2B1    | heterogeneous nuclear ribonucleoprotein A2/B1                               | 133257    | P22626             | 1.634 | 0.0059  | N        | O          |
| HNRNPF       | heterogeneous nuclear ribonucleoprotein F                                    | 1710628   | P52597             | 1.445 | 0.0393  | N        | O          |
| MARCKS       | myristoylated alanine-rich protein kinase C substrate                         | 76803798  | P29966             | 2.018 | 0       | PM       | O          |
| CALR         | calreticulin                                                                 | 117501    | P27797             | 4.181 | 0       | C        | TR         |
| YBX1         | Y box binding protein 1                                                       | 54040030  | P67809             | 1.401 | 0.0297  | N        | TR         |
| NPM1         | nucleophosmin (nucleolar phosphoprotein B23, numatrin)                       | 114762    | P06748             | 1.32  | 0.0201  | N        | TR         |
| HNRNPD       | heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa) | 13124489  | Q14103             | 1.483 | 0.0068  | N        | TR         |
| TUFM         | Tu translation elongation factor, mitochondrial                             | 1706611   | P49411             | 2.205 | 0.0143  | C        | TR         |
| EIF2S1       | eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa           | 124200    | P05198             | 1.474 | 0       | C        | TR         |
| HNRNP1       | heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)     | 254763463 | Q00839             | 1.637 | 0.0252  | N        | Tp         |

*aRatio, OCUM-12/SP ratio: OCUM-12/SP cell compared with parent OCUM-12 cell. OCUM-2MD3/SP ratio: OCUM-2MD3/SP cell compared with parent OCUM-2MD3 cell.
*bLocation: C, cytoplasm; PM, plasma membrane; ES, extracellular space; N, nucleus.
*cType: En, enzyme; K, kinase; Tp, transporter; O, other; TR, transcriptional regulator.

Gastric Cancer Stem Cell Markers by Proteomics Analysis

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Table 2.

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**Figure 2. siRBBP6 transfection into gastric cancer cells.** (A), OCUM-12/SP and OCUM-2MD3/SP showed higher level of RBBP6 mRNA expression than their parent cells, OCUM-12 and OCUM-2MD3. RBBP6 expression in OCUM-12/SP and OCUM-2MD3/SP cells was effectively downregulated by siRBBP6 transfection. (B), Representative images of invading OCUM-12/SP cells showed the number of cancer cells invading the pore membrane filter was decreased by RBBP6 siRNA treatment. siRBBP6 transfection for OCUM12/SP cells significantly inhibited the invasion abilities. Data are presented as the mean and SD (error bars) of four experiments. *p<0.05, **p<0.01. (C), siRBBP6 treatment for OCUM12/SP and OCUM-2MD3/SP cells significantly inhibited the migration abilities, in comparison with that of the control of negative-siRNA treatment. Data are presented as the mean and SD (error bars) of four experiments. *p<0.05, **p<0.01. doi:10.1371/journal.pone.0110736.g002

**Figure 3. Immunohistochemical Determination.** (a) Expression in cancer cells. RBBP6, DCTPP1, and HSPA9 expression were observed primarily in the cytoplasm and nucleus. GLG1, VPS13A, HSPA4, ALDOA, and KRT18 were expressed in the cytoplasm. (b), Expression in epithelial cells. RBBP6, GLG1, VPS13A, DCTPP1, and HSPA9 expression were found some cells in the epithelial gland. KRT18 were expressed in most epithelial cells. HSPA4 and ALDOA expression was not found in normal cells. doi:10.1371/journal.pone.0110736.g003
Table 3. Correlation between proteins expression and clinicopathological variables.

| Factors                          | RBBP6 | GLG1  | VPS13A | DCTPP1 |
|----------------------------------|-------|-------|--------|--------|
|                                  | positive | negative | p-value | positive | negative | p-value | positive | negative | p-value | positive | negative | p-value |
| (n = 151) (n = 143)              | (n = 70) (n = 221) | (n = 149) (n = 147) | (n = 140) (n = 159) |
| T category                       |       |       |       |        |
| T2-T4                            | 102   | 58    | <0.001 | 46     | 115     | 0.045   | 93       | 68       | 0.005   | 105     | 57       | <0.001  |
| (64%) (36%)                      |       |       |       | (29%) (71%) |       |       | (58%) (42%) |       |       | (65%) (35%) |       |       |
| T1                               | 49    | 85    |       | 24     | 106     |       | 56       | 79       |       | 35      | 102      |       |
| (37%) (63%)                      |       |       |       | (19%) (81%) |       |       | (42%) (58%) |       |       | (65%) (35%) |       |       |
| Lymph node metastasis            |       |       |       |        |
| positive                         | 86    | 44    | <0.001 | 34     | 97      | 0.493   | 81       | 50       | <0.001  | 88      | 44       | <0.001  |
| (66%) (34%)                      |       |       |       | (26%) (74%) |       |       | (62%) (38%) |       |       | (67%) (33%) |       |       |
| negative                         | 65    | 99    |       | 36     | 124     |       | 68       | 97       |       | 52      | 115      |       |
| (40%) (60%)                      |       |       |       | (23%) (77%) |       |       | (41%) (59%) |       |       | (31%) (69%) |       |       |
| Distant metastasis               |       |       |       |        |
| positive                         | 40    | 21    | 0.013  | 15     | 46      | 0.912   | 35       | 26       | 0.217   | 41      | 20       | <0.001  |
| (66%) (34%)                      |       |       |       | (25%) (75%) |       |       | (57%) (43%) |       |       | (67%) (33%) |       |       |
| negative                         | 111   | 122   |       | 55     | 175     |       | 114      | 121      |       | 99      | 139      |       |
| (48%) (52%)                      |       |       |       | (24%) (76%) |       |       | (48%) (52%) |       |       | (42%) (58%) |       |       |
| Clinical Stage                   |       |       |       |        |
| I                                | 55    | 89    | <0.001 | 26     | 114     | 0.116   | 58       | 87       | 0.005   | 44      | 103      | <0.001  |
| (38%) (62%)                      |       |       |       | (19%) (81%) |       |       | (40%) (60%) |       |       | (30%) (70%) |       |       |
| II                               | 23    | 24    |       | 14     | 33      |       | 31       | 16       |       | 26      | 22       |       |
| (49%) (51%)                      |       |       |       | (30%) (70%) |       |       | (66%) (34%) |       |       | (54%) (46%) |       |       |
| III                              | 33    | 9     |       | 15     | 28      |       | 25       | 18       |       | 29      | 14       |       |
| (79%) (21%)                      |       |       |       | (35%) (65%) |       |       | (58%) (42%) |       |       | (67%) (33%) |       |       |
| IV                               | 40    | 21    |       | 15     | 46      |       | 35       | 26       |       | 41      | 20       |       |
| (66%) (34%)                      |       |       |       | (25%) (75%) |       |       | (57%) (43%) |       |       | (67%) (33%) |       |       |
| Factors                          | HSPA9 | HSPA4 | ALDOA  | KRT18  |
|                                  | positive | negative | p-value | positive | negative | p-value | positive | negative | p-value | positive | negative | p-value |
| (n = 143) (n = 151)              | (n = 169) (n = 128) | (n = 77) (n = 218) | (n = 127) (n = 163) |
| T category                       |       |       |       |        |
| T2-T4                            | 96    | 65    | <0.001 | 109    | 52      | <0.001  | 50       | 111      | 0.034   | 84      | 76       | 0.001   |
| (60%) (40%)                      |       |       |       | (68%) (32%) |       |       | (31%) (69%) |       |       | (53%) (47%) |       |       |
| T1                               | 47    | 86    |       | 60     | 76      |       | 27       | 107      |       | 43      | 87       |       |
| (35%) (65%)                      |       |       |       | (44%) (56%) |       |       | (20%) (80%) |       |       | (33%) (67%) |       |       |
### Table 3. Cont.

| Factors                     | HSPA9 positive | HSPA9 negative | p-value  | HSPA4 positive | HSPA4 negative | p-value  | ALDOA positive | ALDOA negative | p-value  | KRT18 positive | KRT18 negative | p-value  |
|-----------------------------|----------------|----------------|----------|----------------|----------------|----------|----------------|----------------|----------|----------------|----------------|----------|
|                             | (n = 143)      | (n = 151)      |          | (n = 169)      | (n = 128)      |          | (n = 77)       | (n = 218)      |          | (n = 127)      | (n = 163)      |          |
| Lymph node metastasis       |                |                |          |                |                |          |                |                |          |                |                |          |
| positive                    | 79             | 52             | <0.001   | 97             | 34             | <0.001   | 45             | 86             | 0.004   | 72             | 59             | 0.001   |
| (60%)                       | (40%)          |                |          | (74%)          | (26%)          |          | (34%)          | (66%)          |          | (55%)          | (45%)          |          |
| negative                    | 64             | 99             |          | 72             | 94             |          | 32             | 132            |          | 55             | 104            |          |
| (39%)                       | (61%)          |                |          | (43%)          | (57%)          |          | (20%)          | (80%)          |          | (35%)          | (65%)          |          |
| Distant metastasis          |                |                |          |                |                |          |                |                |          |                |                |          |
| positive                    | 36             | 25             | 0.069    | 44             | 17             | 0.007    | 21             | 40             | 0.096   | 34             | 27             | 0.034   |
| (59%)                       | (41%)          |                |          | (72%)          | (28%)          |          | (34%)          | (66%)          |          | (56%)          | (44%)          |          |
| negative                    | 107            | 126            |          | 125            | 111            |          | 56             | 178            |          | 93             | 136            |          |
| (46%)                       | (54%)          |                |          | (47%)          | (53%)          |          | (24%)          | (76%)          |          | (44%)          | (56%)          |          |
| Clinical Stage              |                |                |          |                |                |          |                |                |          |                |                |          |
| I                           | 49             | 94             | <0.001   | 60             | 86             | <0.001   | 27             | 117            | 0.029   | 48             | 91             | 0.004   |
| (34%)                       | (66%)          |                |          | (41%)          | (59%)          |          | (19%)          | (81%)          |          | (35%)          | (65%)          |          |
| II                          | 31             | 16             |          | 32             | 15             |          | 13             | 34             |          | 19             | 28             |          |
| (66%)                       | (34%)          |                |          | (68%)          | (32%)          |          | (28%)          | (72%)          |          | (40%)          | (60%)          |          |
| III                         | 27             | 16             |          | 33             | 10             |          | 16             | 27             |          | 26             | 17             |          |
| (63%)                       | (37%)          |                |          | (72%)          | (28%)          |          | (37%)          | (63%)          |          | (60%)          | (40%)          |          |
| IV                          | 36             | 25             |          | 44             | 17             |          | 21             | 40             |          | 34             | 27             |          |
| (59%)                       | (41%)          |                |          | (72%)          | (28%)          |          | (34%)          | (66%)          |          | (56%)          | (44%)          |          |

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distant metastasis ($p<0.001$), and stage advancement ($p<0.001$); HSPA9 was significantly associated with invasion depth ($p=0.001$), LN metastasis ($p<0.001$), and stage advancement ($p<0.001$); HSPA4 was significantly associated with invasion depth ($p<0.001$), LN metastasis ($p<0.001$), distant metastasis ($p=0.007$), and stage advancement ($p<0.001$); ALDOA was significantly associated with invasion depth ($p=0.004$), LN metastasis ($p=0.001$), and stage advancement ($p=0.029$); and KRT18 was significantly associated with invasion depth ($p=0.001$), LN metastasis ($p=0.001$), distant metastasis ($p=0.034$), and stage advancement ($p=0.004$).

Prognosis

The Kaplan-Meier plots suggested that of the eight overexpressed proteins, RBBP6, GLG1, VPS13A, DCTPP1, HSPA9, HSPA4, ALDOA, and KRT18, were significantly associated with poor survival in all patients (Figure 4). The cumulative five-year overall survival rate of RBBP6-positive cases (61%) was significantly less ($p = 0.002$) than that of RBBP6-negative cases (78%). Moreover, in patients at stage III, the overall survival rate of RBBP6-positive cases was significantly less ($p = 0.034$) than that of RBBP6-negative cases. The prognosis of patients with DCTPP1-positive tumors (63%) was significantly poorer ($p = 0.016$) than that of DCTPP1-negative tumors (73%). The five-year overall survival rate of HSA4-negative cases (75%). The five-year overall survival rate of ALDOA-positive tumors exhibiting over-expression (61%) was significantly poorer ($p = 0.043$) than that of ALDOA-negative tumors (72%). In contrast, no significant correlations were observed between other proteins and patient survival.

Discussion

Gastric cancer results in a poor prognosis because of frequent metastatic processes, such as LN metastasis and peritoneal metastasis [23]. CSCs have been proposed as having an important
role in the malignant potential of cancer cells, including distant metastasis and chemoresistance [4]. We have since discovered that SP cells obtained from gastric cancer subjects possess these CSC properties [3]. We confirmed that SP cells utilized in this study express candidate gastric cancer stem cell markers including CD44 [24], CD133 [25], and NANOG [26] (Figure S2). The spheroid colony formation activity of these SP cells was higher than that of the parent cells [18]. Also, these CSC-like SP cells display chemoresistance to anticancer drugs [2]. These findings have confirmed that OCUM-12/SP cells and OCUM-2MD3/SP

| Clinicopathological features | Univariate analysis | Multivariate analysis |
|------------------------------|--------------------|----------------------|
|                              | Risk ratio | 95% CI | p-value | Risk ratio | 95% CI | p-value |
| RBBP6 positive vs negative   | 1.964      | 1.275–3.025 | 0.002    | 1.770      | 1.080–2.901 | 0.023 |
| GLG1 positive vs negative    | 1.391      | 0.885–2.186 | 0.152    |
| VPS13A positive vs negative  | 1.145      | 0.885–2.186 | 0.521    |
| DCTPP1 positive vs negative  | 1.661      | 1.096–2.516 | 0.017    | 0.902      | 0.558–1.458 | 0.674 |
| HSFA9 positive vs negative   | 0.983      | 0.747–1.295 | 0.905    |
| HSFA4 positive vs negative   | 1.544      | 1.002–2.378 | 0.049    | 0.509      | 0.349–1.517 | 0.784 |
| ALDOA positive vs negative   | 1.563      | 1.010–2.421 | 0.045    | 1.454      | 0.866–2.442 | 0.156 |
| KRT18 positive vs negative   | 1.390      | 0.919–2.101 | 0.119    |
| Age >60 vs <60               | 1.424      | 0.894–2.268 | 0.137    |
| Sex Male vs female           | 1.065      | 0.680–1.667 | 0.785    |
| Macroscopic type             |           |         |         |
| Type4 vs Other types         | 9.084      | 5.701–14.476 | <0.001 | 4.894      | 2.687–8.914 | <0.001 |
| Tumor differentiation        |           |         |         |
| diffuse vs intestinal        | 1.647      | 1.088–2.500 | 0.018    | 1.155      | 0.679–1.965 | 0.595 |
| T category T2-4 vs T1        | 4.479      | 2.674–7.504 | <0.001 | 1.187      | 0.568–2.479 | 0.648 |
| Vessel invasion positive vs negative | 3.070 | 1.967–4.793 | <0.001 | 0.961      | 0.559–1.653 | 0.886 |
| INF* c vs a & b              | 1.782      | 1.160–2.737 | 0.008    | 0.923      | 0.512–1.666 | 0.791 |
| Hepatic metastasis positive vs negative | 7.776 | 3.369–17.950 | <0.001 | 4.927      | 1.953–12.429 | 0.001 |
| Peritoneal metastasis positive vs negative | 8.209 | 4.734–14.236 | <0.001 | 3.043      | 1.600–5.789 | 0.001 |
| Lymph node metastasis        |           |         |         |
| positive vs negative         | 6.315      | 3.841–10.384 | <0.001 | 2.848      | 1.419–5.717 | 0.003 |
| Total number of resected lymph node |           |         |         |
| <29 vs >30                   | 0.903      | 0.600–1.359 | 0.626    |
| Surgery type D2 vs D1         | 1.886      | 0.589–1.333 | 0.886    |

*INF: Infiltration pattern of tumor. The predominant pattern of infiltrating growth into the surrounding tissue is classified as follows: INF a: The tumor shows expanding growth and a distinct border with the surrounding tissue. INF b: This category is between INF a and INF b. INF c: The tumor shows infiltrating growth and an indistinct border with the surrounding tissue.

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cells may represent cancer stem cell-like properties. Since specific markers for gastric CSCs have not been published as of yet, elucidation of the specific signaling pathways and mechanisms underlying the actions of CSCs might improve the prognosis of gastric cancer. In this study, eight candidate CSCs markers were identified by proteomic techniques using LC-MS/MS coupled with iTRAQ technology. Three proteins, RBBP6, GLG1 and VPS13A, were detected only in SP cell lines but not in their respective parent cell lines. In addition, the five proteins, HSPA9, ALDOA, DCTPP1, HSPA4, and KRT18, were over-expressed in both SP cell lines relative to their respective parent cell lines. RT-PCR analysis also indicated that the expression level of RBBP6, HSPA4, DCTPP1, HSPA9, VPS13A, ALDOA, GLG1, and CK18 was high in OCUM-12/SP and OCUM-2MD3/SP, in compared with the control of parent OCUM-12 and OCUM-2MD3. These proteins were suspected to be novel biomarkers for gastric CSCs. This hypothesis was tested by immunohistochemical analysis of 300 gastric cancer cases.

RBBP6 is a nuclear protein, which is a known to be associated and potentially related to p53, and to retinoblastoma binding Q protein 1 (RBQ1) [27]. RBBP6 interacts with the tumor suppressor proteins p53 and Rb, and plays a role in the induction of apoptosis as well as regulating the cell cycle [28,29]. RBBP6 binds to wild-type p53 proteins but not to p53 mutant [30]. It has also been shown to promote the binding of MDM2 [31], an E3 ubiquitin ligase that targets the p53 [32], and to interfere with its ability to transactivate the target genes [33]. Up-regulation of RBBP6 has been strongly correlated with tumor progression in esophageal cancer and cervical cancer [32]. In this study, RBBP6 expression was associated with T category cancer with regards to invasion depth, distant metastasis, and clinical stage. Moreover, RBBP6 expression was significantly associated with poor survival in patients at all stages, particularly at stage III, resulting in the conclusion that RBBP6 was an independent factor for survival. We performed the RBBP6 siRNA knockdown of RBBP6 gene using OCUM-12/SP and OCUM-2MD3/SP cells in this study. RBBP6 siRNA knockdown significantly decreased the invasion and migration activity of both SP cells, while the sphere forming activity was not different between the negative-siRNA and RBBP6 siRNA in SP cells (data not shown). IHC analysis also indicated that RBBP6 expression was associated with invasion depth, LN metastasis, distant metastasis, and a poor prognosis in gastric cancer patients. RBBP6 might be a novel biomarker of gastric CSCs for clinical diagnosis. These findings suggested that RBBP6 might be closely associated with malignant potential of cancer stem cells, rather than stem cell phenotype.

GLG1 is known as a cysteine-rich fibroblast growth factor receptor (FGFR) [34]. GLG1 was concluded to be associated with the tumorigenesis of some carcinomas [35] and malignancy of brain tumors [36]. FGFR signaling possesses broad mitogenic and cell survival mechanisms, and is involved in a variety of biological processes, including embryonic development, cell growth, and tumor invasion [34]. As observed in this study, GLG1 was concluded to be significantly associated with T category cancer with regards to invasion depth. It should be noted that GLG1 may be associated with invasion potential of CSCs via FGFR signaling.

Vacular protein sorting (VPS) plays a crucial role in the trafficking of molecules between cellular organelles, such as through the trans-Golgi network. The mutation in this gene causes the autosomal disorder characterized by progressive neurodegeneration. A past study found that frameshift mutations of VPS genes, along with loss of expression of VPS13A proteins, are common in gastric cancers with high microsatellite instability and suggests that these alterations may contribute to the development of cancer [37]. VPS is involved in cancer-related cellular mechanisms, such as proliferation [38,39]. As observed in this study, VPS13A was expressed in SP cells and was concluded to be associated with a T category cancer and lymph node metastasis of gastric cancer. It should be noted that VPS13A may be associated with the invasion potential of CSCs.

DCTPP1 is expressed in the nucleus of various cancer cells. Zhang et al. suggested that the accumulation of DCTPP1 in the nucleus of tumor cells might be sufficient for maintaining proper DNA replication needed in order to fulfill the requirement for survival and proliferation of the cells [40]. In conclusion, DCTPP1 was determined to be significantly associated with metastatic activity. It should be noted that DCTPP1 may be associated with the DNA replication of CSCs.

HSPA9 and HSPA4 were concluded to be associated with the invasion and metastatic activity of gastric cancer. These two proteins are members of the heat shock protein HSP-70 family of chaperones, and HSPA9 is the major protein in the mitochondria. Some studies have suggested that HSPA9 and HSPA4 are relevant to cellular apoptosis and promoting proliferation [41,42]. HSPA9 is over-expressed in colon and hepatocellular carcinomas [43,44], while HSPA4 is over-expressed in breast, colon, ovarian, and pancreatic cancers [45]. The HSPA4/HSPA14 axis induces the migration, invasion, and transformation of cancer cells [46]. HSP27 regulates EMT processes and NF-κB activity to contribute to the maintenance of breast CSCs [47]. Inhibition of the HSP70 protein reduced adhesion and induced apoptosis of both acquired and de novo drug resistant cancer cells [48]. The HSPA9 protein is one of the markers of a colon cancer stem cell population [49]. In conclusion, these findings suggest that HSPA9 and HSPA4 are associated with CSCs properties via chaperones for EMT-associated molecules.

ALDOA, aldolase isozymes, is a key glycolytic enzyme that catalyzes the reversible conversion of fructose-1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [50]. Glycolysis is one of the key factors for CSC properties. ALDOA has been expressed in a variety of cancers, such as lung cancer, renal cell and hepatocellular carcinoma [51,52]. As observed in this study, ALDOA was concluded to be significantly associated with the malignant potential of gastric cancer with regards to invasion depth, LN metastasis, and clinical stage. In addition, these findings suggest that ALDOA may be associated with the glycolysis of CSCs.

KRT18 is a type I intermediate filament and its filament partner is keratin 8 (KRT8). KRT18 is involved in intracellular signaling pathways that regulate cell growth [53]. Fortier et al. currently reported that KRT18/KRT8 is associated with the epithelial-mesenchymal transition (EMT) of cancer cells [53]. EMT is ultimately thought to promote tumor progression through the generation of CSC properties [54,55]. These findings suggested that KRT18 may be associated with the EMT of CSCs.

We previously reported on the proteomic differential display analysis of normal gastric mucosal tissues and human gastric carcinoma cell lines, including OCUM-12 and OCUM-2MD3 [56]. Nineteen protein spots were observed to be up-regulated in SGC cell lines when compared to normal gastric mucosa tissues by using 2-DE and LC-MS/MS. Among the identified increased spots, two proteins, including UDP-glucose 6-dehydrogenase and the electron transfer flavoprotein subunit alpha, were also up-
regulated in OCUM-12/SP cells, as opposed to OCUM-12 cells. Three proteins, including nucleophosmin, peroxiredoxin-1, and elongation factor were up-regulated in OCUM-2MD3/SP cells, as opposed to OCUM-2M cells. Conversely, three proteins, 14-3-3 protein sigma, glucosidase 2 subunit beta, and protein DJ-1, were down-regulated in OCUM-12/SP cells as opposed to OCUM-12 cells in this study. Overall, the eight proteins, UDP-glucose 6-dehydrogenase, electron transfer flavoprotein, nucleophosmin, peroxiredoxin-1, elongation factor, 14-3-3 protein sigma, glucosidase 2 subunit beta, and protein DJ-1 may be associated with CSCs as well. To the best of our knowledge, this is the first proteomic analysis that provides evidence that HSPA9, ALDOA, DCTP1, HSPA4, KRT18, RBBP6, GLG1, and VPS13A may be candidate CSC markers for gastric cancer. In particular, RBBP6 may be a promising predictive marker for the prognosis of patients with gastric cancer.

Supporting Information

Figure S1 Representative picture of side population fraction. Cancer cells, which disappear in the presence of verapamil (lower panel), are outlined and defined as the SP cells.

Figure S2 Stem cell markers expression. The expression level of CSC markers, CD44, CD133 and NANOG was significantly higher in OCUM-12/SP (1.6-, 3.6- and 33.2-fold) and OCUM-2MD3/SP cells (2.1-, 5.9- and 3.6-fold) than that in parent OCUM-12 and OCUM-2MD3 cells.

Figure S3 Expression of RBBP6 and ALDOA. The expression level of RBBP6 and ALDOA was high in OCUM-12/SP and OCUM-2MD3/SP cells, in comparison with that of OCUM-12 and OCUM-2MD3 cells.

Table S1 (DOCX)

Table S2 Proteins increased in both OCUM-12/SP and OCUM-2MD3/SP cells.

(DOCX)

Table S3 (DOCX)

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Author Contributions

Conceived and designed the experiments: TM MY AK. Performed the experiments: TM MY AI H. Kinoshita TF. Analyzed the data: TM MY AK AI. Contributed reagents/materials/analysis tools: TM MY AK H. Kasashima TF H. Kinoshita GM KS NK KM MO HW KH. Wrote the paper: TM MY AK HW KH.

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