Original contribution

Clinicopathological significance of claspin overexpression and its association with spheroid formation in gastric cancer☆☆☆

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Summary Gastric cancer (GC) is one of the leading causes of cancer-related death worldwide. Spheroid colony formation is a useful method to identify cancer stem cells (CSCs). The aim of this study was to identify a novel prognostic marker or therapeutic target for GC using a method to identify CSCs. We analyzed the microarray data in spheroid body-forming and parental cells and focused on the CLSPN gene because it is overexpressed in the spheroid body-forming cells in both the GC cell lines MKN-45 and MKN-74. Quantitative reverse-transcription polymerase chain reaction analysis revealed that CLSPN messenger RNA expression was up-regulated in GC cell lines MKN-45, MKN-74, and TMK-1. Immunohistochemistry of claspin showed that 94 (47%) of 203 GC cases were positive. Claspin-positive GC cases were associated with higher T and N grades, tumor stage, lymphatic invasion, and poor prognosis. In addition, claspin expression was coexpressed with CD44, human epidermal growth factor receptor type 2, and p53. CLSPN small interfering RNA treatment decreased GC cell proliferation and invasion. These results indicate that the expression of claspin might be a key regulator in the progression of GC and might play an important role in CSCs of GC.

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1. Introduction

Gastric cancer (GC) is a common type of human cancer, and although therapeutic outcomes have recently improved for early GC, it remains one of the world's leading causes of cancer-related death [1].

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In the past decade, GC has been recognized as a stem cell disease [2]. Cancer stem cells (CSCs) have been suggested to drive tumor initiation and sustain self-renewal [3]. Also, CSCs are closely associated with chemotherapy resistance, recurrence, and metastasis [3]. Therefore, characterizing CSCs is important to establish more effective cancer treatments. One effective method of characterizing CSCs is spheroid colony formation [3-7]. Previously, we performed microarray analyses in spheroid body-forming and parental cells in GC cell lines and reported up-regulation of several kinesin genes including KIFC1 and KIF11 [4]. In the present study, we searched candidate genes from the previous microarray data and found that the CLSPN gene was up-regulated in both the MKN-45 and MKN-74 GC cell lines.

Claspin is a nuclear protein related to DNA replication and damage response and is an important regulator for the S-phase checkpoint [8]. Phosphorylated claspin interacts with checkpoint kinase 1 (CHK1) promoting its activation by ataxia telangiectasia–mutated and Rad3-related kinase (ATR)–dependent phosphorylation [8-10]. Down-regulation of claspin, and ATR and Chk1 greatly reduces cell survival and promotes alterations in cell cycle checkpoints and DNA repair systems [9]. These alterations may lead to genomic instability that triggers cancer development [11-13]. However, overexpression of claspin has also been reported in several human solid tumors such as colon, lung, bladder, breast, and cervical cancers [13-15]. Therefore, previous results indicate that depending on the circumstances, claspin is involved in functions that promote both tumor suppression and cell proliferation. To our knowledge, however, detailed function and expression profiles of the CLSPN gene in human GC remain to be analyzed.

Thus, the present study is the first detailed analysis of claspin expression in GC including its clinicopathological significance and biological function. To clarify the pattern of expression and localization of claspin in GC, we performed immunohistochemical analysis of surgically resected GC samples and investigated the association between claspin and various molecules including CSC markers.

2. Materials and methods

2.1. Tissue samples and cell lines

In this retrospective study, 203 primary tumors were collected from patients diagnosed as having GC who underwent curative resection surgery at Hiroshima University Hospital (Hiroshima, Japan). All samples were obtained with patient consent, and the present study was approved by the Ethical Committee for Human Genome Research of Hiroshima University. Only patients without preoperative radiotherapy or chemotherapy were enrolled in the study. Ninety-eight of 102 patients with stage II/III/IV received chemotherapy after the surgery, but 4 patients who had worse performance status did not receive chemotherapy. The chemotherapy modalities are almost the same during the spanning of the inclusive years. The study population included 124 men and 79 women. Postoperative follow-up was scheduled every 1, 2, or 3 months during the first 2 years after surgery and every 6 months thereafter, unless more frequent follow-up was deemed necessary. Chest x-rays, chest computed tomographic scans, and serum chemistry analyses were performed at every follow-up visit. Recurrence was evaluated from the patient records at Hiroshima University Hospital. Patients were followed up by their physician until the patient's death or date of the last documented contact. Archival formalin-fixed, paraffin-embedded tissues from the 203 patients who had undergone surgical excision for GC were examined using immunohistochemical analysis. All 203 GC cases were histologically classified into differentiated type (well- or moderately differentiated tubular adenocarcinoma and papillary adenocarcinoma) and undifferentiated type (poorly differentiated adenocarcinoma and signet-ring cell carcinoma) according to the Japanese classification of GC. Tumor staging was performed according to the TNM stage grouping system. Written informed consent was not obtained; thus, for strict privacy protection, all identifying information associated with the samples was removed before the analysis.

Human GC–derived cell lines MKN-1, MKN-7, MKN-45, MKN-74, and TMK-1 were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (Whittaker, Walkersville, MD) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

2.2. Quantitative reverse-transcription polymerase chain reaction analysis and Western blotting

Total RNA was extracted using an RNeasy Mini kit (Qiagen, Valencia, CA), and 1 μg of total RNA was converted to cDNA using the First Strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ). Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, CA, USA), as described previously [16]. β-Actin (ACTB gene) was used as an internal housekeeping control. Western blotting was performed as described previously [17].

2.3. Immunohistochemistry

We used archival formalin-fixed, paraffin-embedded tissues from 203 patients who had undergone surgical excision of GC between 2003 and 2007 at Hiroshima University Hospital. One or 2 representative tumor blocks, including the tumor center, invading front, and the tumor-associated nonneoplastic mucosa, from each patient were examined by immunohistochemistry (IHC). For large, late-stage tumors, 2 different
sections were examined to include representative areas of the tumor center and the lateral and deep tumor invasive front. Immunohistochemical analysis was performed using a Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA). Antigen retrieval was performed by microwave heating in citrate buffer (pH 8.0) for 60 minutes.

Fig. 1  Quantitative RT-PCR analysis of CLSPN. A, CLSPN mRNA was measured in MKN-45 and MKN-74 cells. B, CLSPN mRNA expression level in 14 normal tissues and 5 GC cell lines. C, T/N ratio of CLSPN mRNA level between GC tissue (T) and corresponding nonneoplastic mucosa (N) in 14 GC cases. A T/N ratio greater than 2 was considered to represent overexpression. Up-regulation of the CLSPN gene was observed in 5 (35.7%) of 14 GC cases.
Peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub>-methanol for 5 minutes, and the sections were incubated with normal goat serum (Dako Cytomation) for 20 minutes to block nonspecific antibody binding sites. Sections were incubated with a rabbit polyclonal anticlaspin antibody (dilution 1:20 000) for 1 hour at room temperature, followed by incubation with Envision+ antimouse peroxidase for 1 h. The sections were incubated with DAB Substrate-Chromogen Solution (Dako Cytomation) for 5 minutes for color reaction and then were counterstained with 0.1% hematoxylin. Negative controls were created by omission of the primary antibody.

The expression of claspin in GC was scored in all tumors as positive or negative. When more than 5% of tumor cells were stained, immunostaining was considered positive for claspin (according to the median cutoff values rounded off to the nearest 5%). Using these definitions, 2 observers (G. K. and K. S.) without knowledge of clinical and pathologic parameters or the patient outcomes, independently reviewed immunoreactivity in each specimen. If there were slight discrepancies between 2 sections or interobserver differences, it was resolved by consensus review at a double-headed microscope after independent review. The expression of CD44, aldehyde dehydrogenase isoform 1 (ALDH1), CD133, matrix metalloproteinase 7 (MMP7), human epidermal growth factor receptor type 2 (HER2), epidermal growth factor receptor (EGFR), β-catenin, and p53 was scored in all tumors as positive or negative. When more than 10% of tumor cells were stained, the immunostaining was considered positive for each molecule. HER2 IHC score was defined as scores of 0, 1+, 2+, and 3+, following the scoring system of Hofmann et al [18]. All cases with IHC 3+ or IHC 2+ were defined as HER2 IHC positive.

2.4. Fluorescence in situ hybridization

All HER2 IHC 2+ cases were examined by fluorescence in situ hybridization (FISH) using the PathVysion HER2 DNA Probe Kit (PathVysion Kit; Abbott Molecular, Des Plaines, IL) containing a spectrum orange–labeled HER-2 gene (17q11.2-q12) probe and a spectrum green–labeled centromere control for chromosome 17 (17q11.1-q11.1). Analysis was carried out using a Leica CytoVision fluorescence microscope (CytoVision; Leica Biosystems, Nußloch, Germany) equipped with appropriate filters. A minimum of 60 nonoverlapping nuclei were evaluated, and the ratio of HER-2 signals per nuclei relative to chromosome 17 centromere signals were calculated. Ratio scores of greater than 2.0 were classified as HER2 amplification. HER2 IHC 2+ tumors with HER2 amplification or HER2 IHC 3+ were finally considered HER2 positive.

Fig. 2  Immunohistochemical analysis of claspin. A, Claspin expression in differentiated-type GC tissue. B, Claspin expression in undifferentiated-type GC tissue. C, Claspin expression in corresponding nonneoplastic gastric mucosa. D, Kaplan-Meier plot of survival for patients with GC by tumor claspin expression. Anticlaspin antibody immunohistochemical staining, original magnifications ×100 (A and B) and ×40 (C).
2.5. RNA interference

To knock down endogenous claspin, RNA interference (RNAi) was carried out as described previously [19]. Small interfering RNA (siRNA) oligonucleotides for claspin and a negative control were purchased from Invitrogen. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen, CA, USA) according to the manufacturer’s protocol. Briefly, 60 pmol of siRNA and 10 μL of Lipofectamine RNAiMAX were mixed in 1 mL of RPMI medium (10 nmol/L final siRNA concentration). After 20 minutes of incubation, the mixture was added to the cells, and these were plated on dishes for each assay. The cells were analyzed at 48 hours after transfection in all experiments.

2.6. Cell growth and in vitro invasion assays

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to examine cell growth. The cells were seeded at a density of 2000 cells/well in 96-well plates. Cell growth was monitored after 1, 2, and 4 days. Modified Boyden chamber assays were performed to evaluate the invasiveness. The cells were plated at 10,000 cells/well in RPMI 1640 medium plus 1% serum in the upper chamber of a Transwell insert (pore diameter, 8 μm; Chemicon, Temecula, CA) coated with Matrigel. Medium containing 10% serum was placed in the bottom chamber. After 1 and 2 days, the cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained with CyQuant GR dye to assess the number of cells. We performed 3 different experiments and calculated the mean and SD in each of the MTT assays and the Modified Boyden chamber assays.

2.7. Statistical analysis

Correlations between the clinicopathological parameters and claspin expression were analyzed using the Fisher exact test. Kaplan-Meier survival curves were constructed for claspin-positive and claspin-negative patients, and survival rates of the 2 groups were compared. Differences between the survival curves were tested for statistical significance using the
log-rank test. Univariate and multivariate Cox regression analyses were used to evaluate the associations between clinical covariates and survival as described previously [20]. A P value of less than .05 was considered to indicate statistical significance. The SPSS software program (SPSS, Chicago, IL) was used for all statistical analyses.

3. Results

3.1. Messenger RNA expression of CLSPN in the spheroid body–forming GC cells, systemic normal organs, GC cell lines, and GC tissue

To confirm up-regulation of the CLSPN gene in the spheroid body–forming cells, the expression of CLSPN messenger RNA (mRNA) was measured by quantitative RT-PCR in the MKN-45 and MKN-74 cell lines. CLSPN mRNA expression was more than 2-fold higher in the spheroid body–forming cells than in the parental cells in both the MKN-45 and MKN-74 cells (Fig. 1A). Next, to confirm whether the CLSPN gene is cancer specific, quantitative RT-PCR was performed in 5 GC cell lines and in 14 types of normal tissue. CLSPN expression was detected at low levels or to even a lesser extent in various normal organs. However, high CLSPN expression was observed in GC cell lines MKN-45, MKN-74 and TMK-1 (Fig. 1B). Moreover, we analyzed CLSPN expression in 14 GC tissue samples and 14 corresponding nonneoplastic mucosa samples by quantitative RT-PCR. We calculated the ratio of mRNA expression levels between GC tissue (T) and corresponding nonneoplastic mucosa (N). T/N ratios greater than 2 were considered to represent overexpression. CLSPN mRNA was up-regulated in 5 (35.7%) of the 14 cases (Fig. 1C).

3.2. Immunohistochemical analysis of claspin in GC

To analyze the tissue localization, pattern of distribution, and relationship between clinicopathological characteristics and claspin in GC, we performed IHC in the 203 human GC samples. Claspin expression was detected in 94 (47%) of the 203 GCs, and it showed nuclear staining in tumor cells irrespective of the histology (Fig. 2A and B). In the nonneoplastic gastric mucosa, the staining of claspin was either weak or absent in epithelial and stromal cells (Fig. 2C). Next, we analyzed the relationship between claspin expression and various clinicopathological characteristics. Claspin expression was associated with higher T grade (P = .0028), N grade (P = .0045), tumor stage (P = .0003), and lymphatic invasion (P = .0025) in claspin-positive than claspin-negative GC cases (Table 1). Claspin expression was not associated with age, sex, M grade, histology, or venous invasion.
3.3. Relationship between claspin expression and prognosis in GC

We performed a Kaplan-Meier analysis to investigate the association between claspin expression and patient prognosis to further elucidate the clinical impact of claspin on GC in 187 of our patients. Claspin expression was significantly associated with a poorer prognosis \( (P = .0468, \text{log-rank test; Fig. 2D}) \). We also performed univariate and multivariate Cox proportional hazards analyses but did not find claspin expression to be an independent prognostic predictor (data not shown).

3.4. Analysis of the correlation between claspin expression and various molecules including CSC markers

We revealed that claspin could contribute to tumor progression in GC. However, it remains unclear what molecules claspin is associated with. Therefore, we investigated the relationship between claspin expression and various molecules, including some stem cell markers (CD44, ALDH1, CD133), MMP7, β-catenin, p53, HER2, and EGFR. We revealed that claspin expression was coexpressed with CD44 \( (P = .0336) \), HER2 IHC \( (P = .0201) \), and p53 \( (P = .0423; \text{Fig. 3, Table 2}) \). Also, both CD44 and claspin expressions in consecutive tumor sections were observed in regions of lymphatic invasion (Fig. 3Aa and B).

Next, we performed HER2 FISH analysis in all of 20 HER2 IHC 2+ cases. HER2 gene amplification was demonstrated in 8 (40%) of 20 cases, and HER2 positivity including HER2 gene amplification or HER2 IHC 3+ was confirmed in 20 (16%) of 123 cases (Fig. 3D). The result showed a tendency of claspin expression to be associated with HER2 positivity \( (P = .0957; \text{Table 2}) \).

3.5. Effect of claspin down-regulation on cell growth and invasive activity of GC cells

To analyze the biological significance of claspin in GC, siRNA knockdown was performed on the MKN-45 GC cell line and confirmed by Western blot and quantitative RT-PCR (Fig. 4A and B). To investigate the possible antiproliferative effects of CLSPN knockdown, we performed an MTT
Claspin expression in GC

4. Discussion

In this study, we investigated a gene expression profile with GC cell lines that were previously analyzed by microarray analysis [4] and focused on CLSPN as a novel target gene. The rationale for in-depth analysis of CLSPN was based on 3 main reasons. First, CLSPN expression was more than twice higher in spheroid body–forming cells than in parental cells in both MKN-45 and MKN-74 cells. Second, quantitative RT-PCR analysis revealed that CLSPN was more frequently up-regulated in GC tissue than in nonneoplastic gastric mucosa. Third, the expression and biological significance of CLSPN in human GC have not been investigated. The present immunohistochemical analyses showed that claspin expression was associated with T and N grades, tumor stage, lymphatic invasion, and poor prognosis. Furthermore, knockdown of CLSPN by RNAi was found to inhibit cancer cell proliferation and invasion in GC cell lines. Taken together, these results suggested that claspin likely plays an important role in tumor progression.

Immunohistochemical analysis showed that 94 (47%) of the 203 GC cases displayed claspin expression. Also, claspin expression was coexpressed with CD44, HER2, and p53. In GC, CD44 is up-regulated in spheroid formation and is widely used as one of the cell surface markers associated with CSCs [21,22]. Moreover, CD44 expression was reported to significantly correlate with lymphatic invasion and poor survival in GC [23]. Indeed, both CD44 and claspin expression showed
coexpression in regions of lymphatic invasion. The tumor suppressor p53 is a key regulator of the DNA damage response [24], and it also coexpressed with claspin. Mutations of p53 have already been shown to lead to the generation of CSCs [25]. Claspin is reported to be modulated by HERC2/USP20 in coordinating CHK1 activation, leading to genome stability and suppression of tumor growth [26]. When DNA damage occurs, HERC2 dissociates from USP20, resulting in USP20 up-regulation, which in turn stabilizes claspin and promotes the activation of ATR-claspin-CHK1 [27]. In contrast, USP20 itself is considered a tumor suppressor protein [26], whereas CHK1 is involved in promoting tumor growth in a variety of human tumors and its overexpression promotes CSC properties [28,29]. In the present study, claspin expression was also associated with CD44 and the tumor progression. Thus, claspin might induce CSC properties in collaboration with CHK1. Of note, our immunohistochemical analysis showed that the percentage of claspin-positive GC cells was almost 5% to 10%. Because CSCs are minor population of cancer cells [30], claspin might have potential as a marker for gastric CSCs.

Although claspin expression was associated with higher T grade, N grade, tumor stage, and lymphatic invasion, the P value of prognosis was borderline. We speculate that it might be related to analyzing overall survival. There might have been more significant difference if we investigated disease-specific survival rate. Thus, extensive study would be required to clarify the more detailed relationship between claspin expression and prognosis in GC.

To date, there have been no studies in the literature concerning the biological function and role of claspin in GC. In the present study, knockdown of claspin resulted in decreased cell proliferation and invasion in comparison with negative control cells. However, CLSPN knockdown did not significantly affect the levels of EGFR and its downstream molecules. Li et al [31] showed that the inhibitor of both EGFR and HER2 significantly suppressed claspin and induced apoptosis in drug-sensitive breast cancer cells. Indeed, our immunohistochemical results showed that claspin expression significantly correlated with HER2 expression in GC. However, there were no significant association between claspin expression and HER2 positivity including HER2 gene amplification. One of the reasons is speculated to be due to the heterogeneity of HER2 protein in GC, as previously reported that concordance between HER2 IHC 2+ and HER2 amplification is more variable, especially in GC [32]. The present result that claspin positivity was detected more frequently in HER2-positive GC suggests that claspin expression might be an effective predictor in HER2 targeting advanced GC. Further studies are needed in the near future to elucidate the tissue specificity of the detailed signaling pathway involving CLSPN.

The present study showed that claspin might be a promising molecule for treating GC. However, extensive study is required to elucidate the molecular mechanism of its activity in tumor cell biology. Evaluating the molecular mechanism of claspin involvement in tumor cell growth might improve our understanding of GC carcinogenesis and tumor progression.

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