Induction of KIFC1 expression in gastric cancer spheroids

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Abstract. Gastric cancer (GC) is one of the most common human cancers. Spheroid colony formation is an effective model for characterization of cancer stem cells. However, gene expression profiles of spheroid colonies obtained from GC cells have not been examined. We performed microarray analyses by Human Genome U133 Plus 2.0 Array in spheroid body-forming and parental cells from MKN-45 and MKN-74 GC cell lines. Kinesin family member C1 (KIFC1) was expressed >2-fold higher in spheroid body-forming cells than in parental cells in both GC lines. Both the number and size of spheres from MKN-45 cells were significantly reduced upon KIFC1 siRNA-transfection compared with negative control siRNA-transfection. Immunohistochemical analysis of 114 GC tissue samples revealed that 42 (37%) of GC cases were positive for KIFC1 expression. GC cases positive for KIFC1 were found more frequently in stage III/IV cases than in stage I/II cases. GC cases positive for KIFC1 were found more frequently in intestinal type GC cases than in diffuse type GC cases. Furthermore, KIFC1-positive GC cases showed high Ki-67 labeling index. Kaplan-Meier analysis demonstrated that KIFC1 expression was not associated with survival. We found positive expression of KIFC1 in CD44-positive GC and aldehyde dehydrogenase 1 (ALDH1)-positive GC cells. Our results showed that KIFC1 is overexpressed in GC. Since knockdown of KIFC1 inhibited sphere formation, KIFC1 likely plays an important role in cancer stem cells.

Introduction

Gastric cancer (GC) is one of the most common human cancers. Better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment and prevention of GC (1,2). We previously performed serial analysis of gene expression (SAGE) on four primary GC tissues and identified several genes whose expression was either upregulated or downregulated in GC (3). Of these genes, REG4 and OLFM4 were found to encode secreted proteins and serve as highly sensitive serum markers for GC. Although detailed functions of REG4 and OLFM4 are unclear, it has been reported that REG4 is upregulated in aldehyde dehydrogenase 1 (ALDH1)-positive gastric cancer stem cells (CSCs) (4). OLFM4 is a highly specific and robust marker for Lgr5-positive stem cells of small intestine (5), suggesting that both REG4 and OLFM4 play important roles in stem cell function. In the past decade, cancer has been recognized as a stem cell disease (6). CSCs have been described in numerous solid tumors and have been characterized by specific cell surface marker expression including CD44, CD133 and ALDH1 (7). However, gastric CSCs have not been completely characterized. To characterize CSCs, spheroid colony formation is a useful method (8). To form spheroid colony, cells are cultured in culture dishes specially coated for non-attachment with serum-free media. The spheroid colonies have characteristics of CSC phenotype (8). However, gene expression profile of spheroid colonies from the GC cells remains unclear.

In the present study, we analyzed gene expression profile of spheroid colonies from the GC cell lines by microarray analysis, and found that expression of KIFC1 was upregulated in gastric spheroid colonies. KIFC1 (also known as HSET) is a C-type kinesin of the kinesin-14 family (9), and is assumed to be a minus end-directed motor protein (10). Kinesins are a family of molecular motors and play important roles in intracellular transport or cell division (11). Alteration of several types of kinesins have been reported in human cancers (11). Among them, upregulation of KIFC1 has been shown in human glioblastoma, lung, breast and colon cancer (12). In breast cancer cells, forced expression of KIFC1 inhibits docetaxel-mediated apoptosis (13). However, expression of KIFC1 in GC has not been investigated. Therefore, we analyzed the expression and distribution of KIFC1 in human GC by immunohistochemistry, and examined the relationship between KIFC1 staining and clinicopathologic characteristics. We also analyzed the effect of inhibiting KIFC1 expression by RNA interference (RNAi) on the spheroid formation in GC cells.

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Materials and methods

Cell lines. Two cell lines derived from human GC (MKN-45 and MKN-74) were used. All cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All cell lines were maintained in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Spheroid colony formation. For the generation of spheroids, 2,000 cells were plated on a 24-well ultra-low attachment plate (Corning, Corning, NY, USA). Cells were grown in mTeSR medium (StemCell Technologies Inc.). The plates were incubated at 37°C in a 5% CO₂ incubator for 15 days. Sphere number and size were determined and counted under a microscope.

Microarray analysis. Total RNA from MKN-45 spheroid body-forming and parental cells and MKN-74 spheroid body-forming and parental cells was isolated by lysing cells in Isogen lysis buffer (Nippon Gene, Tokyo, Japan) followed by isopropanol precipitation. We performed microarray analyses using the Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) as previously described (14). The arrays were scanned with a GeneChip Scanner 3000 (Affymetrix), and the data were analyzed by Microarray Suite version 5.0 with Affymetrix default analysis settings and global scaling as normalization method. The trimmed mean target intensity of each array was arbitrarily set to 1,000.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Total RNA was extracted with an RNAeasy Minikit (Qiagen, Valencia, CA, USA), and 1 µg of total RNA was converted to cDNA using the First Strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ, USA). Quantitation of KIF2C, KIF4A, KIF11, KIF15, KIF20A, KIF20B, KIF22, KIF23 and KIFC1 mRNA levels was performed by real-time fluorescence detection as previously described (15). PCR was conducted using the SYBR-Green PCR Core Reagents kit (Applied Biosystems). Real-time detection of the emission intensity of SYBR-Green bound to double-stranded DNA was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). ACTB-specific PCR products were amplified from the same RNA samples and served as an internal control.

RNAi and cell growth assay. Short interfering RNA (siRNA) oligonucleotides targeting KIFC1 and a negative control were purchased from Invitrogen (Carlsbad, CA, USA). We used three independent BST2 siRNA oligonucleotide sequences. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) as previously described (16). Briefly, 60 pmol of siRNA and 10 µl of Lipofectamine RNAiMAX were mixed in 1 ml of RPMI-1640 medium (10 nmol/l final siRNA concentration). After 20 min of incubation, the mixture was added to cells, and then cells were plated in culture dishes. Forty-eight hours after transfection cells were analyzed.

To examine cell growth, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. The cells were seeded at a density of 2,000 cells/well into 96-well plates. Cell growth was monitored after 1, 2 and 4 days. Three independent experiments were performed. Mean ± SE was calculated for each of the experiments.

Western blot analysis. Cells were lysed as previously described (17). The lysates (40 µg) were solubilized in Laemmli sample buffer by boiling and then subjected to 10% SDS-polyacrylamide gel electrophoresis followed by electro-transfer onto a nitrocellulose filter. Anti-KIFC1 monoclonal antibody was purchased from Abnova. Peroxidase-conjugated anti-mouse IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection system (Amersham Biosciences). β-actin (Sigma, St. Louis, MO, USA) was also stained as a loading control.

Tissue samples. In a retrospective study design, 114 primary tumors were collected from patients diagnosed with GC who underwent surgery between 2003 and 2007 at Hiroshima University Hospital (Hiroshima, Japan). The present study was approved by the Ethics Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan). All patients underwent curative resection. Only patients without preoperative radiotherapy or chemotherapy and without clinical evidence of distant metastasis were enrolled in the study. Operative mortality was defined as death within 30 days of patients leaving the hospital, and these patients were removed from the analysis. Postoperative follow-up was scheduled every one, two or three months during the first two years after surgery and every six months thereafter unless more frequent follow-up was deemed necessary. Chest X-ray, chest computed tomography scan and serum chemistries were performed at every follow-up visit. Patients were followed by the patients physician until their death or the date of the last documented contact.

Immunohistochemistry. One or two representative tumor blocks, including the tumor center, invading front and tumor-associated non-neoplastic mucosa, was examined from each patient by immunohistochemistry. In cases of large, late-stage tumors, two different sections were examined to include representative areas of the tumor center as well as of the lateral and deep tumor invasive front. Immunohistochemical analysis was performed with a Dako EnVision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpenteria, CA, USA). Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 min. Peroxidase activity was blocked with 3% H₂O₂-methanol for 10 min, and sections were incubated with normal goat serum (Dako Cytomation) for 20 min to block non-specific antibody binding sites. Sections were incubated with a mouse monoclonal anti-KIFC1 (1:50; Abnova) or anti-Ki-67 antibodies (Dako Cytomation) for 1 h at room temperature, followed by incubation with EnVision+ anti-mouse peroxidase for 1 h. For color reaction, sections were incubated with the DAB substrate-chromogen solution (Dako Cytomation) for 10 min. Sections were counterstained with 0.1% hematoxylin. Negative controls were created by omission of the primary antibody.
Expression of KIFC1 was scored in all tumors as positive or negative. When >10% of tumor cells were stained, the immunostaining was considered positive for KIFC1. Using these definitions, two surgical pathologists (N.O. and K.S.), without knowledge of the clinical and pathologic parameters or the patients outcomes, independently reviewed immunoreactivity in each specimen. Interobserver differences were resolved by consensus review at a double-headed microscope after independent review. For the Ki-67-index, a total of 1,000 nuclei were counted to evaluate the percentage of positive nuclei. The Ki-67-index was considered to reflect the proliferative index. Immunostaining of CD44 and ALDH1 was also performed as previously described (7).

Statistical methods. Associations between clinicopathological parameters and KIFC1 expression were analyzed by Fisher’s exact test. Kaplan-Meier survival curves were constructed for KIFC1-positive and KIFC1-negative patients. Survival rates were compared between KIFC1-positive and KIFC1-negative groups. Differences between survival curves were tested for statistical significance by a log-rank test. Differences in the sphere number and size between the two groups were tested by the Student t-test.

Results

Gene expression profile of the spheroid body-forming and the parental cells. To characterize spheroid colonies from the GC cells, we performed microarray analyses by Human Genome U133 Plus 2.0 Array in the spheroid body-forming and the parental cells from MKN-45 cells. We found 797 genes whose expression was significantly higher in the spheroid body-forming cells than in the parental cells, and 646 genes whose expression was significantly lower the spheroid body-forming cells than in the parental cells. Microarray analyses were also performed in the spheroid body-forming and the parental cells from MKN-74 cells. We found 822 genes whose expression was significantly higher in the spheroid body-forming cells than in the parental cells, and 599 genes whose expression was significantly lower than spheroid body-forming cells in the parental cells. To identify ideal biomarkers for gastric CSC, we focused on genes whose expression was high in the spheroid body-forming cells from both MKN-45 and MKN-74 cells, and identified 255 genes. Among these genes, 9 KIF genes (\textit{KIF11}, \textit{KIF15}, \textit{KIF2C}, \textit{KIF20A}, \textit{KIF20B}, \textit{KIF22}, \textit{KIF23}, \textit{KIFC1} and \textit{KIF4A}) were upregulated in the spheroid body-forming cells from both MKN-45 and MKN-74 cells. Kinesins are a family of molecular motors and play important roles in intracellular transport or cell division (11). Alteration of several types of kinesins have been reported in human cancers (11), however, to the best of our knowledge, significance of \textit{KIF} genes in CSC and GC has not yet been studied. Therefore, we decided to investigate \textit{KIF} expression in GC.

Expression of \textit{KIF} genes in the spheroid body-forming cells compared to the parental cells. To confirm upregulation of \textit{KIF} genes in the spheroid body-forming cells, expression of \textit{KIF11}, \textit{KIF15}, \textit{KIF2C}, \textit{KIF20A}, \textit{KIF20B}, \textit{KIF22}, \textit{KIF23}, \textit{KIFC1} and \textit{KIF4A} mRNA was measured by qRT-PCR in MKN-45 and MKN-74 cells (Fig. 1). Among the 9 \textit{KIF} genes, only \textit{KIF11} and \textit{KIFC1} genes were expressed more than twice higher in the spheroid body-forming cells than in the parental cells in both MKN-45 and MKN-74 cells. In the present study, we
focused on KIFC1 since an antibody against KIFC1 protein is commercially available. KIFC1, also known as HSET, is a minus end-directed motor protein that promotes microtubule cross-linking, sliding, bundling and spindle pole focusing, has been identified as an essential mediator of supernumerary centrosome clustering in cancer cells (18). KIFC1 is essential for the survival of cancer cells with extra centrosomes. In contrast, KIFC1 is not essential for mitosis in normal cells, indicating that KIFC1 is a cancer-selective therapeutic target (18). In contrast, significance of KIFC1 in the spheroid body-forming cells has not been studied. Therefore, function of KIFC1 was further analyzed in GC cells.

**Effect of inhibition of KIFC1 on sphere number and size.** Effect of KIFC1 inhibition on sphere number and size was investigated. First, MKN-45 GC cells, in which obvious KIFC1 protein was detected, were selected for these experiments. We examined transition of KIFC1 expression by western blot analysis of cell extracts of MKN-45 cells transfected with KIFC1-specific siRNAs. Three types of siRNAs (siRNA1, siRNA2 and siRNA3) were transfected into MKN-45. The anti-KIFC1 antibody detected a band of ~74 kDa in western blot analyses of cell extracts from MKN-45 GC cells, and the expression of KIFC1 was substantially suppressed by treatment with siRNA1 and siRNA2 (Fig. 2A). Therefore, to knock down the endogenous KIFC1, we used siRNA1 and siRNA2 in the following experiments. We analyzed sphere number and size 15 days after siRNA transfection. The number of spheres from MKN-45 cells was significantly reduced in KIFC1 siRNA1-trasfected and KIFC1 siRNA2-transfected MKN-45 cells than in negative control siRNA-transfected cells (Fig. 2B). The size of sphere from MKN-45 cells was significantly reduced in KIFC1 siRNA1-trasfected and KIFC1 siRNA2-transfected MKN-45 cells compared to negative control siRNA-transfected cells (Fig. 2C). We also analyzed the number and the size of spheres from MKN-74 cells, and similar results were obtained (data not shown). These results suggest that KIFC1 is required for sphere formation in GC cells.

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KIFC1, kinesin family member C1.

Table I. Relationship between KIFC1 expression and clinico-pathological characteristics.
Next, we performed an MTT assay 4 days after siRNA transfection to investigate the antiproliferative effects of KIFC1 inhibition. KIFC1 siRNA1-transfected and KIFC1 siRNA2-transfected MKN-45 cells showed significantly reduced cell growth relative to negative control siRNA-transfected MKN-45 cells (Fig. 2D). We also performed an MTT assay 4 days after siRNA transfection in MKN-74 cells, and similar results were obtained (data not shown). These results indicate that KIFC1 is involved in GC cell growth.

**Immunohistochemical analysis of KIFC1 in GC.** We found that KIFC1 was upregulated in spheroid body-forming cells. In addition, the number and size of spheres were significantly reduced in KIFC1 inhibition. However, expression and distribution of KIFC1 in GC have not been investigated. Therefore, immunohistochemistry was performed on 114 GC tissue samples. In non-neoplastic gastric mucosa, weak or no staining of KIFC1 was observed in the foveolar epithelium or stromal cells. In some cases, nuclear staining was observed in the epithelial cells of gastric isthmus (Fig. 3A), which contains gastric stem cells (19). In contrast, GC tissue showed stronger, more extensive staining. Expression of KIFC1 was frequently observed in intestinal type GC (Fig. 3B). GC cells of diffuse type GC were also stained by KIFC1 (Fig. 3C). Staining of KIFC1 was observed mainly in the nucleus. Many GC cases showed heterogeneity of KIFC1 staining and the percentage of KIFC1-stained GC cells ranged from 0 to 60%. A tendency for upregulation of KIFC1 at the invasive front was not observed. When >10% of tumor cells were stained, the immunostaining was considered positive for KIFC1. In total, 42 (37%) of 114 GC cases were positive for KIFC1.

We next examined the relationship of KIFC1 staining to clinicopathological characteristics (Table I). GC cases positive for KIFC1 were found more frequently in stage III/IV cases than in stage I/II cases. GC cases positive for KIFC1 were found more frequently in intestinal type GC cases than in diffuse type GC cases. Furthermore, KIFC1-positive GC cases showed high Ki-67 labeling index. In contrast, Kaplan-Meier analysis demonstrated that KIFC1 expression was not associated with survival (P=0.0922). Univariate and multivariate Cox proportional hazards analysis also showed that KIFC1 expression was not a prognostic predictor for survival in patients with GC (data not shown).

**Association between KIFC1 expression and CSC marker expression.** Overexpression of KIFC1 was observed in GC. However, it remains unclear whether KIFC1 is associated with gastric CSC. CSC markers include CD133, CD44, CD24, CD166 and ALDH1. Among them, CD44 and ALDH1 are widely used for gastric CSC markers (7). Therefore, immunostaining of CD44 and ALDH1 was also performed in 114 GC cases. As previously reported, in non-neoplastic gastric
mucosa, staining of CD44 was observed in lymphocytes and stromal cells but not epithelial cells. Staining of ALDH1 was detected in the cytoplasm of parietal cells. In GC samples, staining of CD44 and ALDH1 was observed mainly in the membrane and the cytoplasm of the GC cells, respectively. Of 114 GC cases, 53 (46%) GC cases were positive for CD44 and 46 GC cases (40%) were positive for ALDH1. As shown in Fig. 4, expression of KIFC1 was observed in CD44-positive GC and ALDH1-positive GC cells. KIFC1-positive GC cases were significantly frequently found in CD44-positive GC cases (P=0.0001; Fisher’s exact test; Table II). In addition, KIFC1-positive GC cases were significantly frequently observed in ALDH1-positive GC cases (P=0.0007; Fisher’s exact test; Table II).

**Discussion**

To characterize CSCs, one useful method is spheroid colony formation (8). The spheroid colonies have characteristics of CSC phenotype (8). It has been reported that CD44 is upregulated in spheroid formation (20) and CD44 has been identified as one of the cell surface markers associated with CSC (21). These results indicate that genes whose expression is upregulated in spheroid formation are markers for CSC. In the present study, 9 KIF genes (KIF11, KIF15, KIF2C, KIF20A, KIF20B, KIF22, KIF23, KIFC1 and KIF4A) were upregulated in the spheroid body-forming cells by microarray analysis. Among the 9 KIF genes, KIF11 and KIFC1 were expressed more than twice higher in the spheroid body-forming cells than in the parental cells in both MKN-45 and MKN-74 cells. Furthermore, we focused on KIFC1 since an antibody against KIFC1 protein is commercially available. KIFC1 manages spindle length both in mitosis and meiosis using its sliding activity along microtubules. In addition, KIFC1 mediates proper cytokinesis by organizing and stabilizing spindles (22). However, association between KIFC1 and CSC has not been analyzed. In the present study, we showed that both the number and size of spheres from GC cell lines were significantly reduced in KIFC1 siRNA-transfected cells than in negative control siRNA-transfected cells. These results suggest that KIFC1 is required for sphere formation in GC cells.

In non-neoplastic gastric mucosa, nuclear staining of KIFC1 was observed in the epithelial cells of gastric isthmus, which contains gastric stem cells (19). It has been reported that ASPM, which encodes abnormal spindle microtubule assembly, is also expressed in gastric isthmus (23). ASPM is a microtubule minus end-associated protein, and is associated with controlling self-renewal and symmetrical cell division in stem/progenitor cells (24), suggesting that spindle organization by KIFC1 and ASPM plays an important role in gastric stem cells. It is well known that one of characteristics of stem cells is drug-resistance (25). It has been reported that expression of endogenous microtubule depolymerizing factors may favor the development of docetaxel-resistance (26). In fact, overexpression of KIFC1 increases resistance to docetaxel (13). Therefore, expression of KIFC1 can confer resistance to docetaxel in the epithelial cells of gastric isthmus.

In summary, we found that KIFC1 is overexpressed in GC. Since CSC was characterized as a minority population (<5%)
of cells (27), KIFC1 is not a specific marker for gastric CSC. However, since knockdown of KIFC1 by RNAi inhibits sphere formation, KIFC1 likely plays an important role in CSC.

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References


