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Overexpression of KIF11 in Gastric Cancer with Intestinal Mucin Phenotype

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Key Words
KIF11 · Mucin phenotype · Cancer stem cell · Gastric cancer

Abstract
Objective: Gastric cancer (GC) is one of the most common human cancers. A useful method of gastric cancer stem cell (CSC) characterization is spheroid colony formation. Previously, we reported that *KIF11* expression is >2-fold in spheroid-body-forming GC cells compared with parental cells. Here, we analyzed the expression and distribution of KIF11 in human GC by immunohistochemistry. ***Methods:*** Expression of KIF11 in 165 GC cases was determined using immunohistochemistry. For mucin phenotypic expression analysis of GC, immunostaining of MUC5AC, MUC6, MUC2 and CD10 was evaluated. RNA interference was used to inhibit KIF11 expression in GC cell lines. ***Results:*** In total, 119 of 165 GC cases (72%) were positive for KIF11. Expression of KIF11 was not associated with any clinicopathologic characteristics; however, it was observed frequently in GC exhibiting an intestinal phenotype. Both the number and size of spheres formed by MKN-74 cells were significantly reduced following transfection of *KIF11*-targeting siRNA compared with negative-control siRNA. Furthermore, levels of phosphorylated Erk1/2 were lower in *KIF11* siRNA-transfected cells than with negative-control siRNA-transfected cells. ***Conclusion:*** These results indicate that KIF11 is involved in intestinal mucin phenotype GC.

Introduction

Gastric cancer (GC) is one of the most common human cancers. Multiple genetic and epigenetic alterations are associated with GC, and enhanced knowledge of the gene expression changes that occur during gastric carcinogenesis may facilitate improvements in disease diagnosis, treatment and prevention [1]. Genes encoding transmembrane/secretory proteins that are expressed specifically in cancers are ideal diagnostic biomarkers. Moreover, if such a gene product functions in the neoplastic process, the gene is not just a biomarker but may also be a therapeutic target [2].

In the past decade, cancer has been recognized as a stem cell disease [3]. Cancer stem cells (CSCs) have been described in numerous solid tumors. Gastric CSCs are characterized by the expression of specific cell surface markers including CD44, CD133 and aldehyde dehydrogenase 1 (ALDH1) [4]. However, gastric CSCs are incom-

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pletely characterized at present. A useful method to further characterize gastric CSCs is spheroid colony formation [5]. Previously, we have reported that *KIF11* and *KIFC1* expression is >2-fold in spheroid-body-forming cells when compared with parental cells in both MKN-45 and MKN-74 GC cell lines [6]. We also showed that KIFC1 protein expression is upregulated in 37% of GC cases, and that both the number and size of spheres from GC cell lines are significantly reduced in *KIFC1* siRNA-transfected cells compared with negative-control siRNA-transfected cells. These results suggest that KIFC1 is required for sphere formation in GC cells. However, the significance of KIF11 in gastric CSCs has not been studied.

KIF11 protein, also known as Eg5 protein or kinesin spindle protein (KSP), is a plus-end directed heterotetrameric motor protein capable of simultaneously moving along 2 microtubules [7]. KIF11 is overexpressed in human cancers including breast, lung, ovarian, bladder and pancreatic cancer. Furthermore, because KIF11 is not expressed in the adult peripheral nervous system, KIF11 inhibitors may not cause neuropathic side effects. Inhibitors of KIF11 have been developed as chemotherapeutic agents for the treatment of cancer [8]. Filanesib (ARRY-520) is a highly selective, targeted inhibitor of KIF11 that induces mitotic arrest and subsequent tumor cell death. In a phase 1 clinical study, ARRY-520 provided exposures with acceptable tolerability and evidence of target-specific pharmacodynamic effects [8]. There is a possibility that ARRY-520 has activity in patients with GC; however, prior to this study, the expression of KIF11 in GC has not been evaluated.

We analyzed the expression and distribution of KIF11 in human GC using immunohistochemistry, and examined the relationship between KIF11 positivity and clinicopathologic characteristics. Furthermore, because gastric or intestinal mucin phenotypes of GC have distinct clinical characteristics and exhibit specific genetic and epigenetic changes [1], we investigated associations between KIF11 expression and gastric/intestinal mucin phenotypes. We also analyzed the effect of inhibiting KIF11 expression by RNA interference (RNAi) on spheroid formation by GC cells.

Materials and Methods

Tissue Samples

In a retrospective study, samples from 178 primary tumors were collected from patients diagnosed with GC who underwent surgery between 2003 and 2007 at Hiroshima University Hospital (Hiroshima, Japan). This study was approved by the Ethical Committee for Human Genome Research of Hiroshima University, Hiroshima. All patients underwent curative resection. Only patients without preoperative radiotherapy or chemotherapy and no clinical evidence of distant metastasis were enrolled in the study. Operative mortality was defined as death within 30 days of leaving the hospital, and these patients were removed from the analysis. 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-ray, chest computed tomography and serum chemistries were performed at every follow-up visit. Patients were followed by their physicians until their death or the date of the last documented contact.

For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), 13 GC samples were randomly collected. These were frozen immediately in liquid nitrogen and stored at –80°C until use. Fourteen types of normal-tissue samples (heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, leukocytes, spleen, skeletal muscle, brain and spinal cord) were purchased from Clontech.

For immunohistochemical analysis, archival formalin-fixed, paraffin-embedded tissues from 165 patients who had undergone surgical excision for GC were consecutively collected. One or 2 representative tumor blocks, including the tumor center, invading front and tumor-associated nonneoplastic mucosa, from each patient were examined using immunohistochemistry. In cases of large, late-stage tumors, 2 different sections were examined to include representative areas of the tumor center and lateral and deep-tumor invasive fronts. The Ki-67 labeling index was analyzed as described previously [6].

Tumor staging was determined according to the TNM classification system [9]. Histological classification was carried out according to the Japanese Research Society for Gastric Cancer [10].

qRT-PCR Analysis

Total RNA was extracted with an RNeasy mini kit (Qiagen), and 1 μg of total RNA was converted to cDNA using the first-strand cDNA synthesis kit (Amersham Biosciences). Quantitation of *KIF11* mRNA levels was performed by real-time fluorescence detection as described previously [11]. 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.

Immunohistochemistry

Immunohistochemical analysis was performed with a Dako EnVision+ mouse peroxidase detection system (DakoCytomation). Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 min. Peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, and sections were incubated with normal goat serum (DakoCytomation) for 20 min to block nonspecific antibody binding sites. Sections were incubated with a mouse monoclonal anti-KIF11 antibody (1:50, Abcam) for 1 h at room temperature, followed by incubation with EnVision+ anti-mouse peroxidase for 1 h. For a 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 KIF11 was scored in all tumors as positive or negative. When >10% of tumor cells were stained, the immunostaining was considered positive for KIF11. Using these definitions, 2 surgical pathologists (N.O. and K.S.), with no knowledge of the clinical and pathologic parameters or the outcome for patients, independently reviewed immunoreactivity in each specimen. Interobserver differences were resolved by consensus review at a double-headed microscope after independent review.

Phenotypic Analysis

GCs were classified into 4 phenotypes, the gastric (G), intestinal (I), gastric and intestinal mixed (GI) and null (N) types. For phenotypic expression analysis of GC, we performed immunohistochemical analysis (as described above) with 4 antibodies, all from Novocastra: anti-MUC5AC as a marker of foveolar epithelial cells in the stomach, anti-MUC6 as a marker of pyloric gland cells in the stomach, anti-MUC2 as a marker of goblet cells in the small intestine and colorectum and anti-CD10 as a marker of microvilli of absorptive cells in the small intestine and colorectum. The criteria for the classification of G and I type GCs have been described previously [1]. Briefly, GCs in which >10% of cells in the section expressed at least 1 gastric epithelial cell marker (MUC5AC or MUC6) or intestinal epithelial cell marker (MUC2 or CD10) were classified as G or I type cancer, respectively. Sections that showed both gastric and intestinal phenotypes were classified as GI type, and those that lacked both the gastric and intestinal phenotypes were classified as N type.

Cell Lines

Four cell lines derived from human GC (MKN-1, MKN-7, 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 (Nissui Pharmaceutical Co., Ltd.) containing 10% fetal bovine serum (BioWhittaker) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

RNA Interference

Short-interfering RNA (siRNA) oligonucleotides targeting *KIF11* and a negative control were purchased from Invitrogen. We used 3 independent *KIF11* siRNA oligonucleotide sequences. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) as described previously [12]. Briefly, 60 pmol of siRNA and 10 μl of Lipofectamine RNAiMAX were mixed in 1 ml of RMPI medium (10 nmol/l final siRNA concentration). After 20 min of incubation, the mixture was added to the cells and then cells were plated in culture dishes. Forty-eight hours after transfection, cells were analyzed.

Western Blot Analysis

Cells were lysed as described previously [13]. The lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 10% SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose membrane. Anti-KIF11 monoclonal antibody was purchased from Abcam. Anti-Erk1/2 and phospho-ERK1/2 (pErk1/2) antibodies were purchased from Cell Signaling Technology. Peroxidase-conjugated anti-mouse or rabbit IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western blot detection system (Amersham Biosciences). β-Actin (Sigma) was also stained as a loading control.

Spheroid Colony Formation

For the generation of spheres, 2,000 cells were plated per well on 24-well ultra-low attachment plates (Corning). Cells were grown in mTeSR medium (STEMCELL Technologies Inc.). The plates were incubated at 37°C in a 5% CO_2 incubator for 15 days. Sphere number and size were determined and counted under a microscope.

Statistical Methods

Associations between clinicopathologic parameters and KIF11 expression were analyzed by Fisher's exact test. Kaplan-Meier survival curves were constructed for KIF11-positive and KIF11-negative patients. Survival rates were compared between KIF11-positive and KIF11-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 2 groups were tested by Student's t test.

Results

Expression of KIF11 mRNA in Normal Organs and GC

We previously showed a >2-fold expression of *KIF11* in spheroid-body-forming cells compared with parental cells in both the MKN-45 and MKN-74 cell lines. We now measured the mRNA expression levels of *KIF11* in 14 samples of normal organs and 13 GC tissue samples using qRT-PCR to investigate the specificity of *KIF11* expression. Among the 14 normal-organ tissues, *KIF11* mRNA expression was clearly detected in stomach, small intestine, pancreas and bone marrow samples. However, *KIF11* expression was much higher in GC samples compared with these normal tissues (fig. 1a).

Immunohistochemical Analysis of KIF11 in GC

We confirmed that *KIF11* exhibited higher expression in GC compared with normal tissues using qRT-PCR. However, the expression levels and distribution pattern of KIF11 protein in GC have not been previously investigated. Therefore, we conducted immunohistochemical analysis of KIF11 on 165 GC tissue samples. qRT-PCR revealed obvious *KIF11* mRNA expression in a noncancerous stomach, small intestine, pancreas, and bone marrow. Therefore, we first performed immunohistochemical analysis of a noncancerous stomach. Staining of KIF11 was observed in some foveolar epithelial cells (fig. 1b), so the noncancerous stomach served as a positive control.

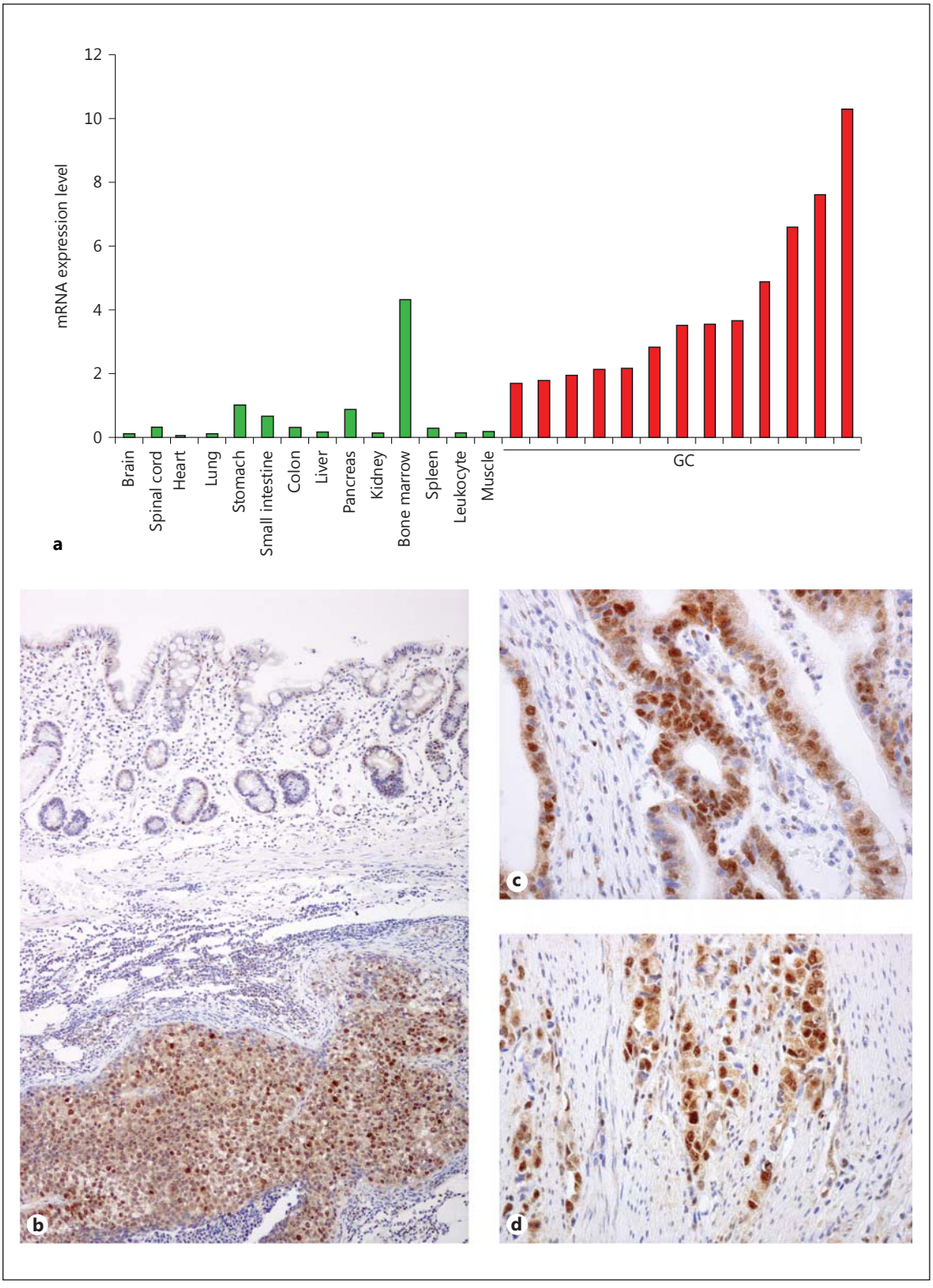


Fig. 1. Expression of KIF11 in GC. **a** qRT-PCR analysis of *KIF11* in various human normal and GC tissues. The units are arbitrary and *KIF11* expression was calculated by standardization of a normal stomach as 1.0. **b** Immunohistochemical analysis of KIF11 in GC and corresponding nonneoplastic gastric mucosa. ×100. **c** Immunohistochemical analysis of KIF11 in well-differentiated type GC. ×400. **d** Immunohistochemical analysis of KIF11 in poorly differentiated type GC. ×400.

Table 1. Relationships between KIF11 expression and clinicopathologic characteristics

	KIF11 expression		p value
	positive	negative	
Age			
<66 years	63 (75%)	21	0.4877
≥66 years	56 (69%)	25	
Sex			
Male	72 (71%)	30	0.5977
Female	47 (75%)	16	
T classification			
T1	50 (70%)	22	0.5998
T2/3/4	69 (74%)	24	
N classification			
N0	60 (71%)	25	0.7292
N1/2/3	59 (74%)	21	
M classification			
M0	97 (73%)	36	0.6634
M1	22 (69%)	10	
Stage			
Stage I	58 (71%)	24	0.7307
Stage II/III/IV	61 (73%)	22	
Lymphovascular invasion			
Positive	67 (72%)	26	1.0000
Negative	52 (72%)	20	
Vascular invasion			
Positive	48 (67%)	24	0.2203
Negative	71 (76%)	22	
Histologic classification			
Well-differentiated	54 (67%)	27	0.1646
Poorly differentiated	65 (77%)	19	
KIFC1 expression			
Positive	51 (74%)	18	0.7265
Negative	68 (71%)	28	
Ki-67 labeling index			
<40%	56 (68%)	26	0.3013
≥40%	63 (76%)	20	

Table 2. Relationships between KIF11 expression and gastric/intestinal phenotype marker

	KIF11 expression		p value
	positive	negative	
MUC5AC			
Positive	62 (71%)	25	0.8627
Negative	57 (73%)	21	
MUC6			
Positive	12 (67%)	6	0.5850
Negative	107 (73%)	40	
MUC2			
Positive	34 (83%)	7	0.1070
Negative	85 (69%)	39	
CD10			
Positive	13 (72%)	5	1.0000
Negative	106 (72%)	41	

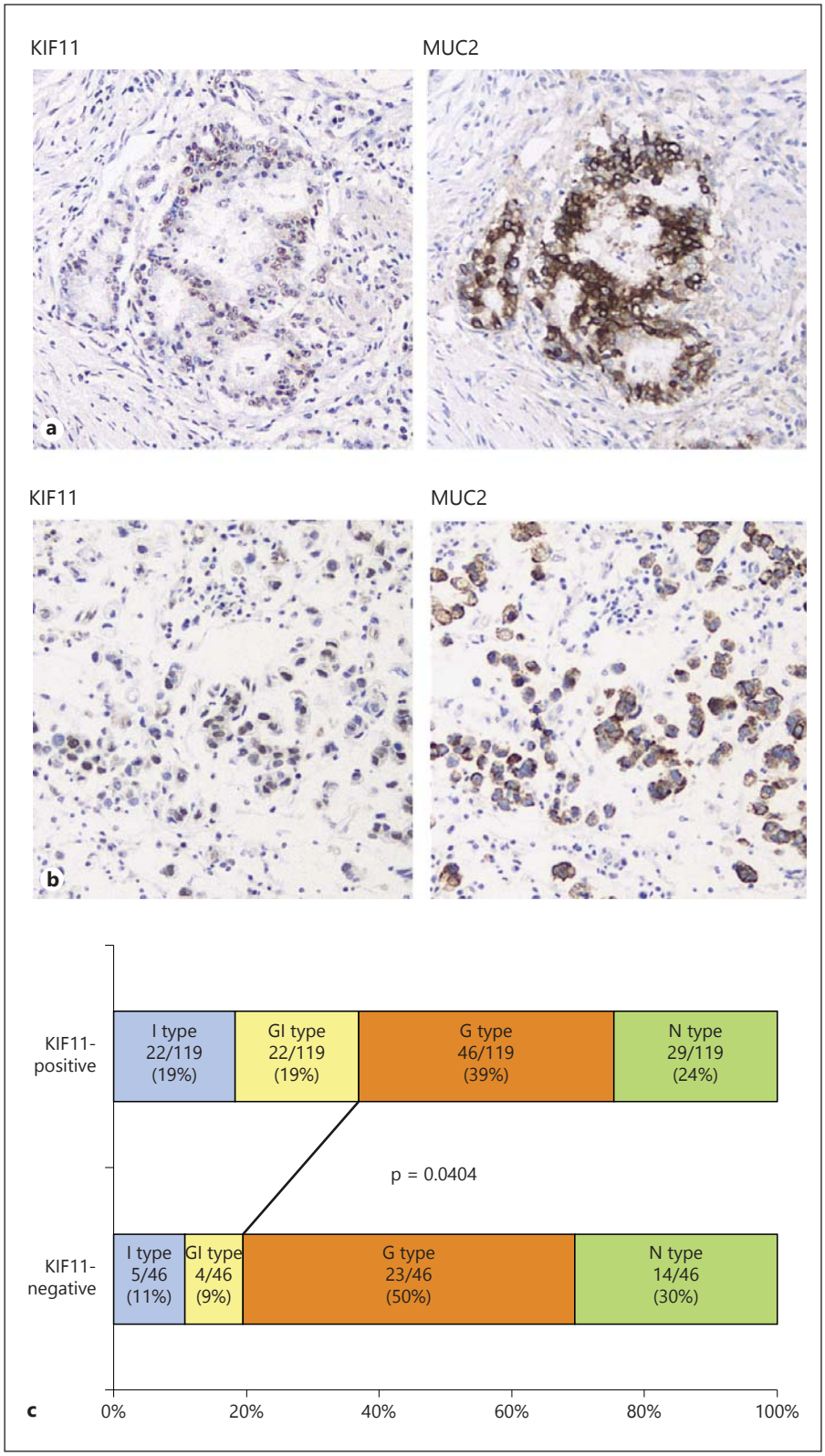
In contrast, GC tissue showed stronger, more extensive staining (fig. 1b). Staining of KIF11 was observed in both well-differentiated (fig. 1c) and poorly differentiated GC (fig. 1d). KIF11 signals were predominately nuclear. Many GC cases exhibited heterogeneity of KIF11 staining, and the percentage of KIF11-stained GC cells ranged from 0 to 90%. A tendency for upregulation of KIF11 at the invasive front was not observed. When >10% of tumor cells were stained, the sample was considered positive for KIF11 expression. In total, 119 of 165 GC cases (72%) were positive for KIF11.

We next examined relationships between KIF11 staining and clinicopathologic characteristics (table 1). KIF11 expression was not associated with any clinicopathologic characteristics. Furthermore, the Kaplan-Meier analysis demonstrated that KIF11 expression was not associated with survival (data not shown). Univariate and multivariate Cox proportional hazards analyses showed that KIF11 expression was not a prognostic predictor for survival in patients with GC (data not shown). We have previously reported that KIFC1 expression is >2-fold in spheroid-body-forming cells compared with parental cells, and that KIFC1 protein expression is upregulated in 37% of GC cases [6]. Therefore, we also compared expression of KIF11 with that of KIFC1. No association between KIF11 and KIFC1 expression was identified (table 1).

We also analyzed relationships between KIF11 staining and clinicopathologic characteristics using other cut-off points. When >50% of tumor cells were stained, the immunostaining was considered positive for KIF11. In total, 65 of 165 GC cases (39%) were positive for KIF11, and similar results were obtained (data not shown). For phenotypic expression analysis of GC, we performed immunohistochemical analysis with 4 gastric or intestinal markers. When >10% of tumor cells were stained, the sample was considered positive for gastric or intestinal markers. Therefore, for KIF11 analysis, when >10% of tumor cells were stained, the sample was considered positive in the following experiments.

Association between KIF11 Expression and Phenotype Marker

GC can be subdivided into 4 phenotypes (G, I, GI and N types) on the basis of mucin expression [1]. We



Color version available online

Fig. 2. Expression of the mucin phenotype of GC. **a** Expression of KIF11 and MUC2 in well-differentiated type GC. ×400. **b** Expression of KIF11 and MUC2 in poorly differentiated type GC. ×400. **c** Summary of KIF11 expression and expression of the mucin phenotype.

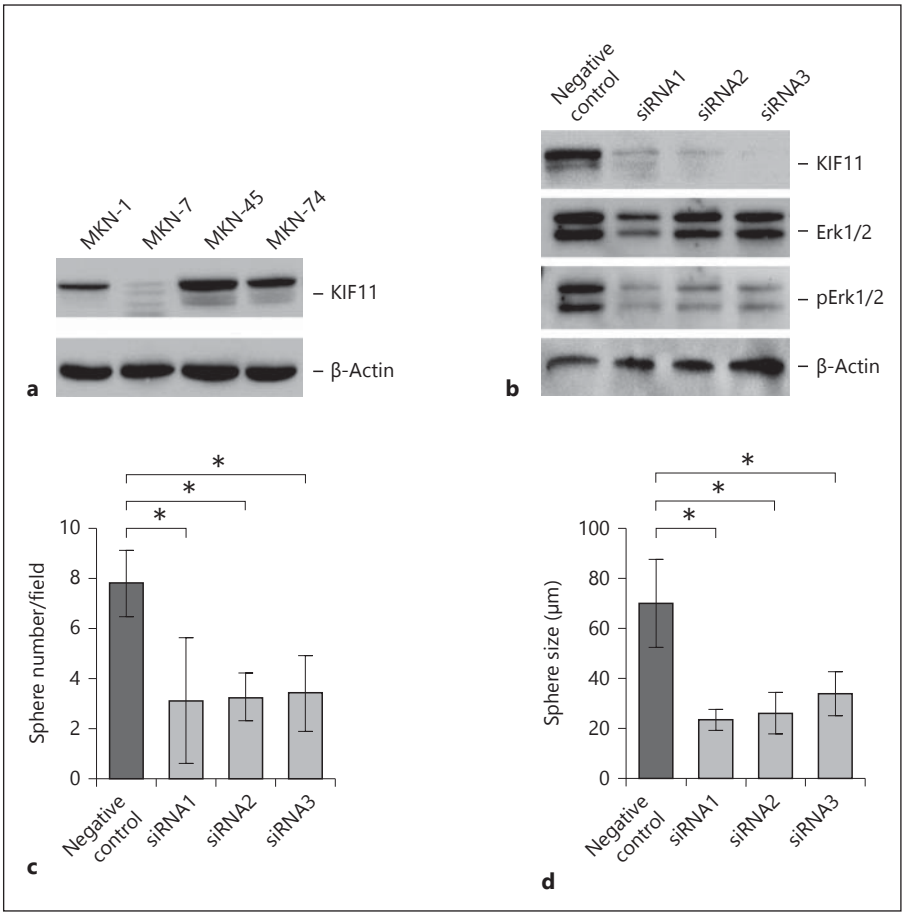


Fig. 3. Effect of KIF11 inhibition in GC cells. **a** Western blot analysis of KIF11 protein levels in 4 GC cell lines. **b** Western blot analysis of KIF11, Erk1/2 and phospho-Erk1/2 (pErk1/2) protein levels in cell lysates from MKN-74 cells transfected with *KIF11* siRNA or negative-control siRNA. β-Actin levels were measured as a loading control. **c** The number of spheres from MKN-74 cells transfected with *KIF11* siRNA or negative-control siRNA. Bars and error bars indicate the mean and SD, respectively, of 3 independent experiments. * $p < 0.05$. **d** The size of spheres from MKN-45 cells transfected with *KIF11* siRNA or negative-control siRNA. Bars and error bars indicate the mean and SD, respectively, of 3 independent experiments. * $p < 0.05$.

investigated associations between KIF11 expression and the mucin phenotype, because gastric or intestinal mucin phenotypes of GC have distinct clinical characteristics and exhibit specific genetic and epigenetic changes. Immunohistochemical analysis of gastric (MUC5AC and MUC6) and intestinal (MUC2 and CD10) markers was conducted on 165 GC tissue samples. KIF11 was frequently expressed in MUC2-positive GC cases in both well-differentiated (fig. 2a) and poorly differentiated GC (fig. 2b). KIF11 expression was identified more frequently in MUC2-positive (34/41, 83%) than in MUC2-negative GC (85/124, 69%). However, KIF11 was also expressed in GC cells that did not express MUC2. There was no clear relationship between the expression of KIF11 and that of MUC5AC, MUC6 or CD10 (table 2). We used the expression of these 4 markers to phenotypically classify 69 of the GC cases (42%) as G type, 27 (16%) as I type, 26 (16%) as GI type and 43 (26%) as N type. Expression of KIF11 was observed more frequently in GC exhibiting I phenotypes (I type and GI type GC) than in other GC (G type and N type GC) ($p = 0.0404$; Fisher's exact test; fig. 2c).

Effect of Inhibition of KIF11 on Sphere Number and Size

We found that KIF11 was upregulated in GC samples showing an I phenotype; however, the significance of KIF11 expression in gastric CSCs remained unclear. Therefore, we investigated the effect of *KIF11* inhibition on sphere number and size. We conducted Western blot analysis of KIF11 protein levels in 4 GC cell lines. The anti-KIF11 antibody detected a band of approximately 120 kDa on Western blots of cell extracts from MKN-1, MKN-45 and MKN-74 GC cells (fig. 3a). We next examined the transition of KIF11 expression by Western blot analysis of cell extracts of MKN-74 cells transfected with *KIF11*-specific siRNAs. Three different siRNA sequences (siRNA1–3) were used, and all substantially suppressed the expression of *KIF11* (fig. 3b), so we used all 3 in subsequent experiments. We evaluated the number and size

of spheres 15 days after siRNA transfection. The number (fig. 3c) and size (fig. 3d) of spheres formed by MKN-74 cells transfected with siRNA1, siRNA2 or siRNA3 were significantly reduced compared with those formed by negative-control siRNA-transfected cells. We also analyzed the number and size of spheres formed by MKN-45 cells in identical experiments and obtained similar results (data not shown). These results suggest that KIF11 is required for sphere formation in GC cells.

Phosphorylation of extracellular signal-regulated kinase (Erk) phosphorylation is detectable at the bottom of the small intestinal crypts in which intestinal stem cells are found [14]. The Erk signaling pathway also plays a prominent role in maintaining the stem-like phenotype of rhabdomyosarcoma cells [15]. Therefore, we investigated the effect of *KIF11* inhibition on Erk1/2 phosphorylation. Levels of phosphorylated Erk1/2 were lower in MKN-74 cells transfected with siRNA1, siRNA2 or siRNA3 compared with negative-control siRNA-transfected cells (fig. 3b). Similar results were obtained from experiments using MKN-45 (data not shown).

Discussion

We have previously reported that *KIF11* and *KIFC1* are expressed at higher levels in the spheroid-body-forming cells compared with parental GC cells [6]. We have also shown that KIFC1 protein expression is upregulated in 37% of GC cases [6]. We now analyzed KIF11 expression in GC. Although weak or no staining of KIF11 was observed in nonneoplastic gastric mucosa, 72% of the GC cases were positive for KIF11 on immunohistochemistry. These results suggest that KIF11 plays an important role in GC. Furthermore, we showed that both the number and size of spheres from GC cell lines were significantly reduced in *KIF11* siRNA-transfected cells compared with negative-control siRNA-transfected cells, indicating that KIF11 is required for sphere formation in GC cells. Consistently, inhibition of KIF11 using a small-molecule inhibitor stopped the growth of treatment-resistant glioblastoma tumor-initiating cells [16]. We also showed that the levels of phosphorylated Erk were reduced in *KIF11*-knockdown cells. Taken together, these results suggest that KIF11 likely participates in CSC formation. While KIF11 is a plus-end directed heterotetrameric motor protein capable of simultaneously moving along 2 microtubules in metaphase [7], a complete understanding of the function of KIF11 in CSC and sphere formation remains elusive. Further study is required to clarify its significance.

We found that 72% of examined GC cases were positive for KIF11; however, expression of KIF11 was not associated with TNM stage or histologic classification. In contrast, KIF11 expression was observed more frequently in GC with the I phenotype than in other GC. This suggests that KIF11 plays an important role in I phenotype GC.

KIF11-specific inhibitors promote activation of the spindle checkpoint, mitotic arrest and subsequent cell death in certain cancer cell lines [8]. ARRY-520 is a highly selective, targeted inhibitor of KIF11 that induces mitotic arrest and subsequent tumor cell death. An initial human phase 1 study of ARRY-520 has been conducted in patients with advanced solid tumors, with ARRY-520 providing exposures with acceptable tolerability and evidence of target-specific pharmacodynamic effects [8]. Because 72% of GC cases were positive for KIF11, a KIF11 inhibitor such as ARRY-520 could be effective in patients with GC. Our findings demonstrate that KIF11 is more frequently expressed in GC that exhibits the I phenotype, suggesting that a KIF11 inhibitor could be particularly effective in patients with this form of GC.

In summary, we have identified KIF11 overexpression in GC. KIF11 is not a specific marker of gastric CSCs, as CSCs are a minority population (<5%) of cells [17] while the percentage of KIF11-stained GC cells ranged from 0 to 90% in this study. However, because knockdown of KIF11 by RNAi inhibits sphere formation, KIF11 likely plays an important role in CSC.

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Disclosure Statement

The authors declare no conflict of interest.

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