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KIF11 Is Required for Spheroid Formation by Oesophageal and Colorectal Cancer Cells

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Abstract. *Background: Oesophageal squamous cell carcinoma (ESCC) and colorectal cancer (CRC) are common types of human cancer. Spheroid colony formation is used to characterize cancer stem cell (CSCs). In the present study, we analyzed the significance of kinesin family 11 (KIF11 in human ESCC and CRC. Materials and Methods: Expression of KIF11 in 105 ESCC and 100 CRC cases was determined using immunohistochemistry. RNA interference was used to inhibit KIF11 expression in ESCC and CRC cell lines. Results: In total, 61 out of 105 (58%) ESCC and 62 out of 100 (62%) CRC cases were positive for KIF11. Expression of KIF11 was not associated with any clinicopathological characteristics. Both the number and size of spheres produced by from TE-5 ESCC cells and DLD-1 CRC cells were significantly reduced upon KIF11 siRNA transfection compared to negative control siRNA transfection. Conclusion: These results indicate that KIF11 plays an important role in CSCs of ESCC and CRC.*

Gastrointestinal (GI) cancer, including oesophageal squamous cell carcinoma (ESCC), gastric cancer (GC), and colorectal cancer (CRC), are common malignancies worldwide. A variety of genetic and epigenetic alterations are associated with GI cancer, and better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment, and prevention (1). Genes encoding transmembrane/secretory proteins that are specifically expressed in cancer are ideal diagnostic biomarkers. Moreover, if the gene product functions in the neoplastic process, the gene is not just a potential 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) are defined as malignant cells that possess the ability to initiate tumour growth and sustain self-renewal (4). Moreover, CSCs play an important role in resistance to chemotherapy (4). Therefore, characterization of CSCs is important for establishing more effective cancer treatments. One useful method for characterizing CSCs is spheroid colony formation. We previously showed that *KIF11* and kinesin family C1 (*KIFC1*) genes are more highly expressed, by more than two-fold, in spheroid-forming cells than in the parental cells of GC cell lines (5). We also showed that KIF11 protein expression is up-regulated in GC tissue samples, and that both the number and size of spheres produced by GC cells are significantly reduced by inhibition of *KIF11* (6). These results suggest that *KIF11* likely plays an important role in gastric CSCs.

KIF11 (also known as EG5) is a member of the kinesin superfamily. The kinesin superfamily proteins are classified as mitotic kinesins, which are involved in cell division, and non-mitotic kinesins, which are principally involved in intracellular transport (7). *KIF11* is a mitotic kinesin and is required for the separation of duplicated centrosomes during spindle formation (8). Thus, a *KIF11* inhibitor is thought to be useful to specifically target proliferating tumour tissue (9). Several small molecule *KIF11* inhibitors have been reported (10). There is a possibility that *KIF11* inhibitors

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may exhibit antitumour activity in patients with GI cancer. Despite the potential connection between KIF11 and cancer, the significance of KIF11 in ESCC and CRC has not been characterized.

In the present study, we analyzed the expression and distribution of *KIF11* in human ESCC and CRC by immunohistochemistry, and examined the relationship between *KIF11* expression and clinicopathological characteristics of patients with ESCC, and CRC. Furthermore, we also analyzed the effect of inhibiting *KIF11* expression by RNA interference (RNAi) on spheroid formation in ESCC and CRC cells.

Materials and Methods

Tissue samples. In a retrospective study design, 113 primary tumours were collected from patients diagnosed with ESCC and 113 primary tumours were collected from patients diagnosed with CRC who underwent surgery at Hiroshima University Hospital (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 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 tomographic scan and serum chemistry analysis were performed at every follow-up visit. Patients were followed by their physician until death or the date of the last documented contact.

Tumour staging was determined according to the TNM classification system (11). Histological classification of classifications of ESCC and CRC were based on the World Health Organization system (12,13). This study was approved (no. IRINHI66) by the Ethical Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. For qRT-PCR, eight ESCC samples and 13 CRC samples were examined. Samples were frozen immediately in liquid nitrogen and stored at −80˚C until use. Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA.), and 1 μg of total RNA was converted to cDNA using First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). Quantitation of *KIF11* mRNA level was performed by real-time fluorescence detection as described previously (6). 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). Actin-beta (*ACTB*)-specific PCR products were amplified from the same RNA samples and served as an internal control.

Immunohistochemistry. We used archival formalin-fixed, paraffin-embedded tissues from a total of 205 patients who had undergone surgical excision either for ESCC (n=105) or for CRC (n=100). In addition, 20 colorectal adenoma cases obtained by endoscopic mucosal resection were collected. Immunohistochemical analysis was performed with the Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation). 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. Sections were incubated with a mouse monoclonal antibody to KIF11 (1:50, Abcam) or antibody to aldehyde dehydrogenase 1 family member A1 (ALDH1) (1:200; BD Biosciences, BD Biosciences; San Diego, CA, USA) for 1 h at room temperature, followed by incubation with Envision+ anti-mouse peroxidase for 1 h. For colour reaction, sections were incubated with DAB Substrate-Chromogen Solution (Dako Cytomation, Carpinteria, CA, USA) for 10 min. Sections were counterstained with 0.1% haematoxylin. Negative controls were created by omission of the primary antibody. Expression of KIF1 and ALDH1 was scored in all tumours as positive or negative; when more than 10% of tumour cells were stained, immunostaining was considered positive.

Sections were incubated with the following antibody dilutions; primary anti-CD133 antibody (AC133; Miltenyi Biotec, Auburn, CA, USA) 1:100, anti-ALDH1 anti-body (BD Biosciences; San Diego, CA, USA) 1:200, and anti-CD44 antibody (Novocastra; Newcastle, UK). Sections were incubated with primary antibody for 1 h at room tem-perature. Sections were incubated with the following antibody dilutions; primary anti-CD133 antibody (AC133; Miltenyi Biotec, Auburn, CA, USA) 1:100, anti-ALDH1 anti-body (BD Biosciences; San Diego, CA, USA) 1:200, and anti-CD44 antibody (Novocastra; Newcastle, UK). Sections were incubated with primary antibody for 1 h at room temperature. Sections were incubated with the following antibody dilutions; primary anti-KIF11 antibody (1:50, Abcam), anti-ALDH1 anti-body (1:200, BD Biosciences). Sections were incubated with primary antibody for 1 h at room temperature.

Cell lines. Four cell lines derived from human oesophageal cancer (TE-1, TE-5, TE-8, and TE-9) and five cell lines derived from human CRC (COLO320, Lovo-JC, DLD-1, CCK, and WiDr) 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, Tokyo, Japan) containing 10% foetal bovine serum (BioWhittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

RNAi. Short interfering RNA (siRNA) oligonucleotides targeting *KIF11* and a negative control were purchased from Invitrogen (Carlsbad, CA, USA). We used three independent *KIF11* siRNA oligonucleotide sequences. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) as described previously (14). 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 cells and then cells were plated in culture dishes. Forty-eight hours after transfection, cells were analysed for spheroid colony formation.

Spheroid colony formation. For the generation of spheres, 2×10^3 cells (transfected with *KIF11* siRNA, or negative control siRNA) were plated on 24-well ultra-low attachment plates (Corning, New York, NY, USA). Cells were grown in mTeSR medium (STEMCELL Technologies Inc., Cambridge, MA, USA). The plates were incubated at 37˚C in a 5% CO_2 incubator for 15 days. Sphere number and size were then determined using a microscope.

Table I. *Relationship between kinesin family 11 KIF11 expression and clinicopathological characteristics in oesophageal squamous cell carcinoma.*

Characteristic	Subgroup	KIF11 expression, n (%)		*p*-Value
		Positive	Negative	
Age	<65 Years	25 (56)	20 (44)	0.6478
	≥65 Years	36 (60)	24 (40)	
Gender	Male	7 (47)	8 (53)	0.3326
	Female	54 (60)	36 (40)	
T classification	T1	26 (52)	24 (48)	0.2582
	T2/3/4	34 (63)	20 (37)	
N classification	N0	22 (47)	25 (53)	0.0413
	N1/2/3	38 (66)	19 (34)	
Stage	I	17 (47)	19 (53)	0.1158
	II/III/IV	43 (63)	25 (37)	
Lymphovascular invasion	Negative	39 (62)	24 (38)	0.3326
	Positive	22 (53)	20 (47)	
Vascular invasion	Negative	43 (54)	36 (46)	0.1846
	Positive	18 (69)	8 (31)	
Tumour differentiation	Well or moderately	42 (55)	35 (45)	0.2215
	Poorly	19 (68)	9 (32)	
Ki-67 expression (n=100)	<30%	36 (59)	25 (41)	0.8017
	≥30%	24 (62)	15 (38)	
ALDH1 expression	<10%	29 (62)	18 (38)	<0.0001
	≥10%	54 (93)	4 (7)	

ALDH1: Aldehyde dehydrogenase 1 family member A1.

Western blot analysis. *KIF11* siRNA-transfected, and non-transfected cells were lysed as described previously (15). The lysates (40 μg) were solubilized in Laemmli buffer by boiling and then subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. Monoclonal antibody to KIF11 was purchased from Abcam (Cambridge, MA, USA). 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 used as a loading control.

Statistical methods. Associations between clinicopathological parameters and KIF11 expression were analysed 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 two groups (*KIF11* siRNA-transfected cells and negative control siRNA-transfected cells) were tested by Student *t*-test.

Results

Expression of KIF11 mRNA in ESCC tissue and non-neoplastic mucosa samples. We first analyzed mRNA expression of *KIF11* in eight ESCC tissue samples and non-neoplastic mucosa samples by qRT-PCR (Figure 1A). Overexpression of *KIF11* (tumour/non-neoplastic mucosa ratio >2) was observed in six out of the eight ESCC tissue samples.

The expression and distribution of KIF11 protein in ESCC has not been investigated to our knowledge. Therefore, we performed immunohistochemical analysis of KIF11 in 105 ESCC tissue samples. In non-neoplastic mucosa, weak or no staining of KIF11 was observed in the epithelial cells. In contrast, ESCC tissue showed stronger, more extensive KIF11 staining than non-neoplastic mucosa (Figure 1B). Staining of KIF11 was observed mainly in the nuclei. The percentage of KIF11-stained ESCC cells in the samples ranged from 0% to 90%. We classified immunohistochemical staining as KIF11-positive when more than 10% of tumour cells were stained for KIF11. In total, 61 (58%) of 105 ESCC cases were positive for KIF11.

Next, we analyzed whether KIF11 is associated with CSCs. CSC markers include cluster of differentiation (CD)133, CD44, CD24, CD166, and ALDH1, and among these, ALDH1 is widely used (16). Therefore, we performed immunostaining of ALDH1 in 105 ESCC cases. In the ESCC samples, ALDH1 staining was observed mainly in the ESCC cells. Among the 105 ESCC cases, 54 (51%) were positive for ALDH1. As shown in Figure 1C, expression of KIF11 was observed frequently in ALDH1-positive ESCC cells. Indeed, Fisher's exact test demonstrated that KIF11-positivity in ESCC was significantly frequently associated with ALDH1 positivity ($p<0.0001$; Table I).

We next examined the relationship of KIF11 staining to clinicopathological characteristics in ESCC cases (Table I).

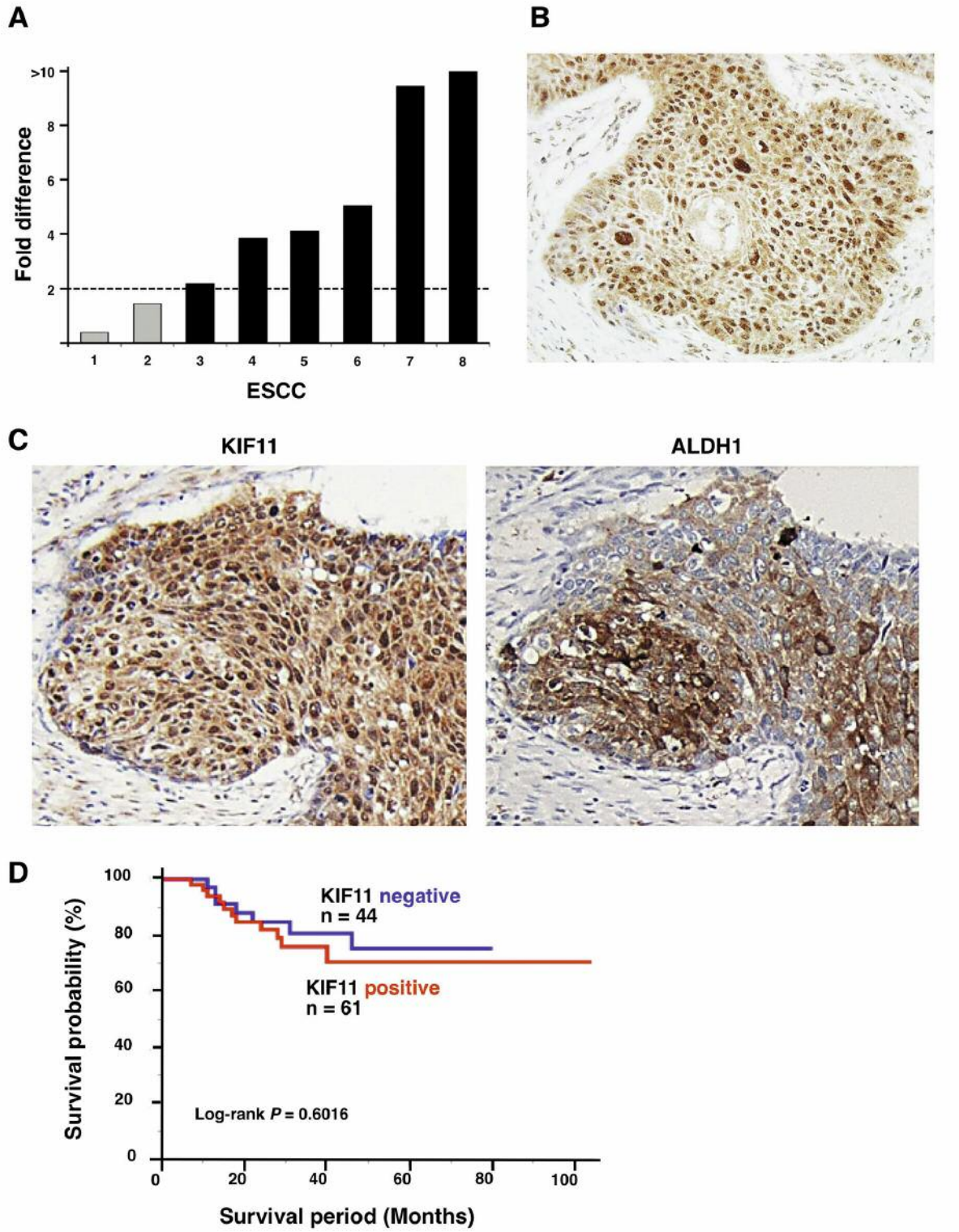

Figure 1. *Expression of kinesin family 11 (KIF11) in oesophageal squamous cell carcinoma (ESCC) tissues. A: Quantitative reverse transcription-polymerase chain reaction analysis of KIF11 in eight ESCC samples. The bars represent individual samples. Fold difference is the ratio of KIF11 mRNA level in ESCC to that in corresponding non-neoplastic mucosa. B: Immunohistochemical analysis of KIF11 in ESCC; original magnification, ×400. C: Immunohistochemical analysis of KIF11 (left) and ALDH1 (right) in consecutive tumour sections of ESCC, showing marked coexpression; original magnification, ×400. D: Kaplan–Meier plot of survival for patients with ESCC by tumour KIF11 expression.*

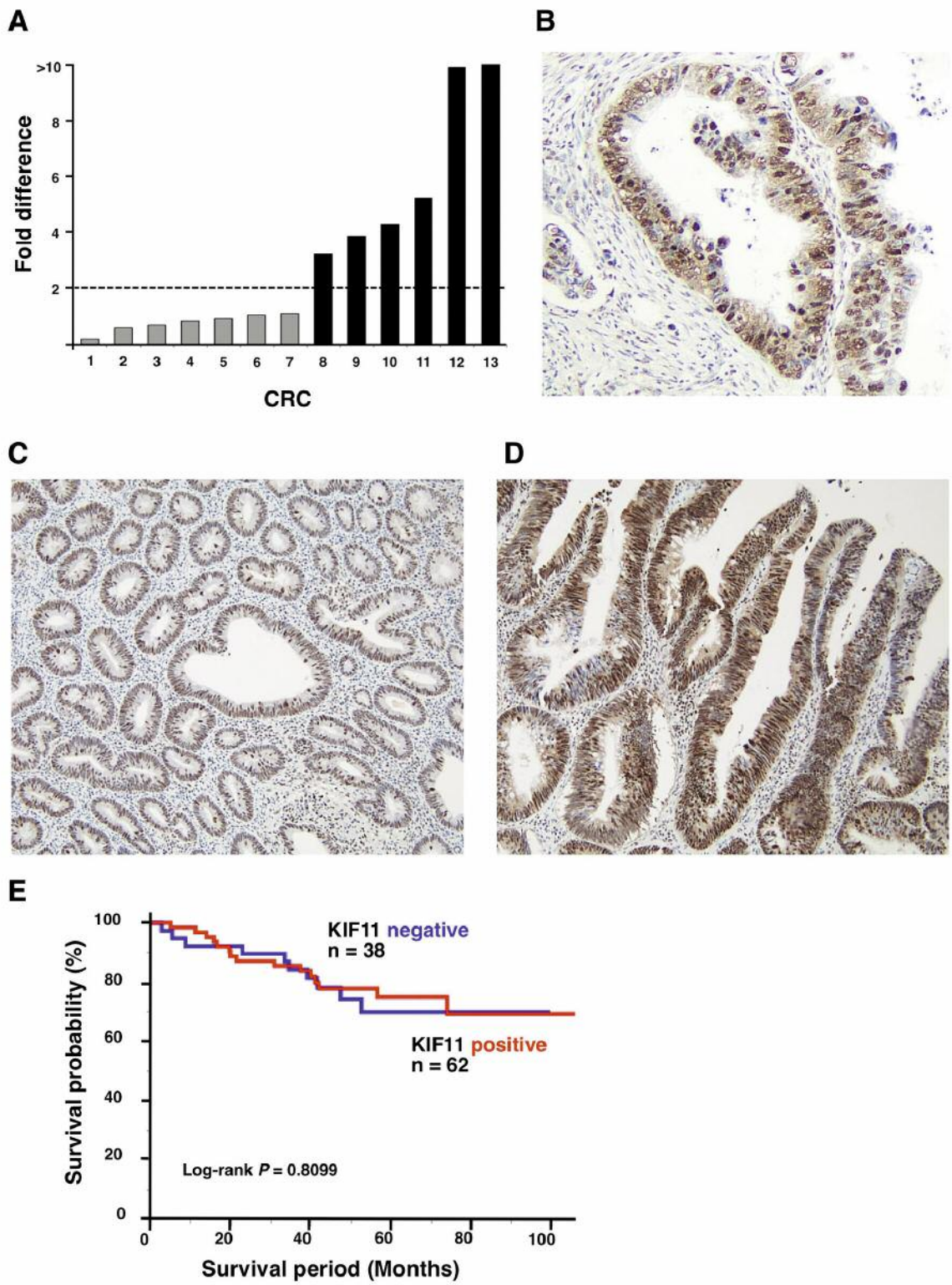

Figure 2. *Expression of kinesin family 11 (KIF11) in colorectal cancer (CRC) tissues. A: Quantitative reverse transcription-polymerase chain reaction analysis of KIF11 in 13 CRC samples. The bars represent individual samples. Fold difference is the ratio of KIF11 mRNA level in CRC to that in corresponding non-neoplastic mucosa. B: Immunohistochemical analysis of KIF11 in CRC; original magnification, ×400. C, D: Immunohistochemical analysis of KIF11 in low-grade (C) and high-grade (D) adenoma; original magnification, ×100. E: Kaplan–Meier plot of survival in patients with CRC by tumour KIF11 expression.*

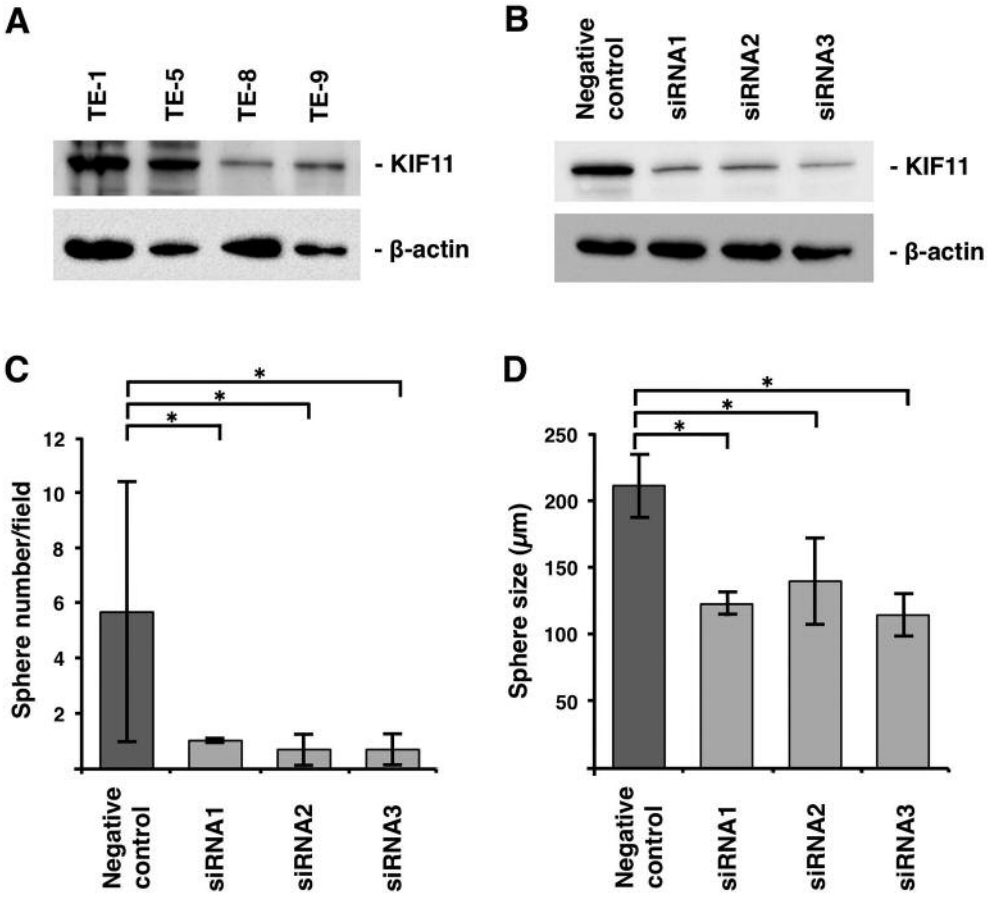

Figure 3. *Effect of kinesin family 11 (KIF11) inhibition in oesophageal squamous cell carcinoma (ESCC) cells. A: Western blot analysis of KIF11 in four ESCC cell lines. B: Western blot analysis of KIF11 in cell lysates from TE-5 cells transfected with KIF11 siRNA or negative control siRNA. β-Actin was included as a loading control. Number (C) and size (D) of spheres produced by TE-5 ESCC cells transfected with KIF11 siRNA or negative control siRNA. Forty-eight hours after transfection, cells were incubated at 37˚C in a 5% CO_2 incubator for 15 days for spheroid colony formation. Bars and error bars indicate the mean and SD, respectively, of three experiments. *Significantly different at $p<0.05$.*

Expression of KIF11 was associated with N classification. Kaplan–Meier analysis demonstrated that KIF11 expression was not significantly associated with survival (Figure 1D). Univariate and multivariate Cox proportional hazards analysis also showed that KIF11 expression was not a prognostic predictor for survival in patients with ESCC (data not shown). These results suggest that KIF11 plays an important role in the pathogenesis of ESCC, but not its progression.

Expression of KIF11 mRNA in CRC tissue and non-neoplastic mucosa samples. We also analyzed expression of KIF11 in CRC. We first performed qRT-PCR analysis of *KIF11* mRNA in 13 CRC tissue samples and non-plastic tissue samples (Figure 2A). Overexpression of *KIF11* (tumour/non-neoplastic mucosa ratio >2) was detected in six out of 13 (46%) CRC tissue samples.

The expression and distribution of KIF11 protein in CRC has also not been investigated as far as we are aware. Therefore, we performed immunohistochemical analysis of KIF11 in 100 CRC tissue samples. In non-neoplastic mucosa, weak or no staining of KIF11 was observed in the epithelial cells. In contrast, CRC tissue showed stronger, more extensive staining than non-neoplastic mucosa (Figure 2B). KIF11 staining was observed mainly in nuclei. In total, 62 out of 100 (62%) CRC cases were positive for KIF11.

We also performed immunohistochemical analysis of KIF11 in 10 low-grade (Figure 2C) and 10 high-grade (Figure 2D) colorectal adenoma cases. In total, eight out of 10 low-grade and nine out of 10 high-grade adenoma cases were positive for KIF11. These results suggest that overexpression of KIF11 is an early event in the pathogenesis of CRC.

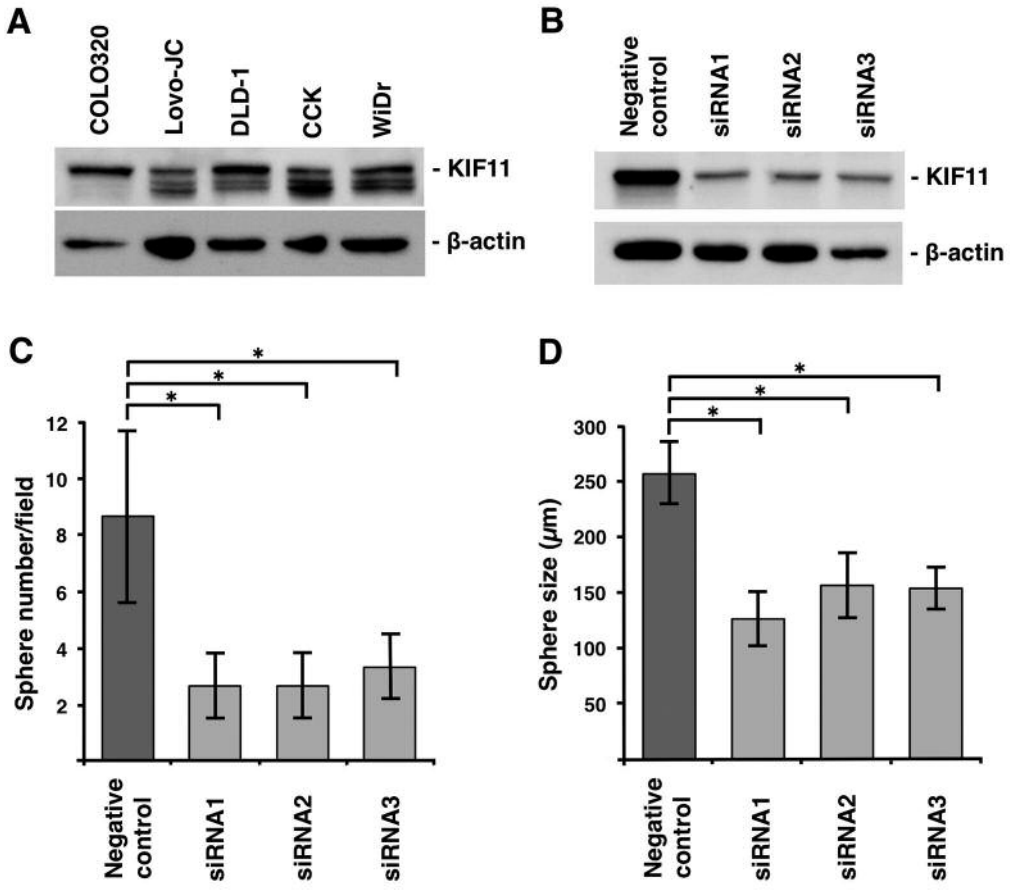

Figure 4. *Effect of kinesin family 11 (KIF11) inhibition in colorectal cancer (CRC) cells. A. Western blot analysis of KIF11 in five CRC cell lines. B: Western blot analysis of KIF11 in cell lysates from DLD-1 cells transfected with KIF11 siRNA or negative control siRNA. β-Actin was included as a loading control. Number (C) and size (D) of spheres produced by produced by DLD-1 CRC cells transfected with KIF11 siRNA or negative control siRNA. Forty-eight hours after transfection, cells were incubated at 37˚C in a 5% CO_2 incubator for 15 days for spheroid colony formation. Bars and error bars indicate the mean and SD, respectively, of three different experiments. *Significantly different at $p<0.05$.*

We next examined the relationship of KIF11 staining to clinicopathological characteristics (Table II). Expression of KIF11 was associated with T classification and histological classification. Kaplan–Meier analysis demonstrated that KIF11 expression was not significantly associated with survival (Figure 2E). Univariate and multivariate Cox proportional hazards analysis demonstrated that KIF11 expression was not a prognostic predictor for survival in patients with CRC (data not shown).

Effect of inhibition of KIF11 on sphere number and size. Our findings demonstrated that *KIF11* was up-regulated in ESCC and CRC tissues. However, the significance of *KIF11* expression in oesophageal and colorectal CSC remains unclear. Therefore, we examined the effect of *KIF11* inhibition on sphere number and size.

We first performed western blot analysis of KIF11 in four ESCC cell lines and confirmed that the antibody to KIF11 detected a band of approximately 120 kDa in all ESCC cell lines (Figure 3A). We next analysed the effect of three siRNAs targeting KIF11 (siRNA1, siRNA2, and siRNA3) by transfecting each siRNA into TE-5 cells, and confirmed that the expression of endogenous *KIF11* was substantially suppressed by all three siRNAs (Figure 3B). Therefore, we used siRNA1, siRNA2, and siRNA3 in subsequent experiments to knock-down endogenous *KIF11* expression. We analyzed sphere number and size in TE-5 cells 15 days after siRNA transfection. The number of spheres was significantly reduced in *KIF11* siRNA-transfected TE-5 cells compared with the negative control siRNA-transfected cells (Figure 3C). The size of spheres was also significantly reduced in KIF11 siRNA-transfected TE-5 cells compared

Table II. *Relationship between kinesin family 11 KIF11 expression and clinicopathological characteristics in colorectal cancer.*

Characteristic	Subgroup	KIF11 expression, n (%)		*p*-Value
		Positive (%)	Negative	
Age	<65 Years	31 (60)	21 (40)	0.6091
	≥65 Years	31 (65)	17 (35)	
Gender	Male	41 (67)	20 (33)	0.1792
	Female	21 (54)	18 (46)	
T Classification	T1	3 (27)	8 (73)	0.0119
	T2/3/4	59 (66)	30 (34)	
N Classification	N0	32 (58)	23 (42)	0.3845
	N1/2/3	30 (67)	15 (33)	
Stage	I	14 (52)	13 (48)	0.2035
	II/III/IV	48 (66)	25 (34)	
Tumour location	Right colon	10 (59)	7 (41)	0.7671
	Left colon	52 (63)	31 (37)	
Tumour differentiation	Well or moderately	62 (65)	34 (35)	0.0091
	Other	0 (0)	4 (100)	

with the negative control siRNA-transfected cells (Figure 3D). Similar results were obtained using TE-1 cells (data not shown).

We performed similar experiments in CRC cell lines. We first detected KIF11 expression in five CRC cell lines by western blot analysis (Figure 4A) and confirmed that the three siRNAs were able to effectively knock-down *KIF11* in DLD-1 CRC cells (Figure 4B). We analyzed sphere formation in DLD-1 cells knocked-down for *KIF11* and found that the number and size of spheres was significantly reduced in *KIF11* siRNA-transfected DLD-1 cells compared to negative control siRNA-transfected cells (Figure 4C and D). These results suggest that *KIF11* is indeed required for sphere formation in ESCC and CRC cells.

Discussion

Previously, we reported that KIF11 protein expression is up-regulated in 72% of GC cases and that KIF11 is involved in spheroid formation (6). In the present study, we analyzed *KIF11* expression in ESCC and CRC tissues. Although weak or no staining of KIF11 was observed in non-neoplastic oesophageal mucosa and colorectal mucosa, 58% of ESCC cases and 61% of CRC cases were positive for KIF11 by immunohistochemistry. Furthermore, expression of KIF11 was not associated with TNM stage or patient survival. Taken together, these results indicate that KIF11 plays an important role in the pathogenesis of ESCC and CRC but not its progression.

We previously demonstrated that *KIF11* and *KIFC1* genes are more highly expressed in spheroid-forming cells than in parental GC cells (5). We also reported that *KIF11* likely plays an important role in gastric CSCs (6). In the present study, we found that both the number and size of spheres produced by ESCC and CRC cell lines were significantly reduced by *KIF11* siRNA transfection compared with negative control siRNA-transfected cells, indicating that *KIF11* is required for sphere formation of ESCC and CRC cells. Moreover, we found that KIF11-positivity in ESCC was significantly frequently associated with ALDH1 positivity by immunohistochemistry. Taken together, these results suggest that *KIF11* is likely to help in the production of CSC in ESCC and CRC.

Mitotic kinesins such as KIF11 are involved in cell division and non-mitotic kinesins are principally involved in intracellular transport. KIF11 is required for the separation of duplicated centrosomes during spindle formation (8). *KIF11* inhibitors have entered phase 1 and 2 clinical trials either as monotherapies or in combination with other drugs (7). *KIF11* is targeted by one of the most advanced mitotic kinesin inhibitors, filanesib (also known as ARRY-520). Filanesib is a highly selective, targeted inhibitor of *KIF11* that induces mitotic arrest and subsequent tumour cell death. A first-in-human phase 1 study in patients with advanced solid tumours has been completed and the results concluded that filanesib provides exposure with acceptable tolerability and evidence of target-specific pharmacodynamic effects (17). In our study, 58% of ESCC cases and 61% of CRC cases were positive for *KIF11* by immunohistochemistry, indicating that *KIF11* inhibitors, such as filanesib, might be effective in patients with ESCC or CRC. *KIF11* inhibition leads to the activation of the spindle checkpoint, mitotic arrest and subsequent cell death in certain cancer cell lines (17). Inhibition of KIF11 was found to stop the growth of

treatment-resistant glioblastoma tumour-initiating cells (18). Therefore, inhibition of *KIF11* may have positive effects in treatment-resistant ESCC or CRC.

In summary, we found that *KIF11* is overexpressed in ESCC and CRC. KIF11 likely plays an important role in ESCC and CRC. Furthermore, our results showed that knockdown of *KIF11* by siRNA inhibits sphere formation, indicating that *KIF11* is important in activity of oesophageal and colorectal CSCs.

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

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