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Original article

Overexpression of KIFC1 and its association with spheroid formation in esophageal squamous cell carcinoma

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ABSTRACT

Esophageal squamous cell carcinoma (ESCC) is one of the most common human cancers. We previously reported that *KIFC1* is involved in gastric cancer pathogenesis and that *KIFC1* plays an important role in gastric cancer spheroid colony formation. However, the significance of KIFC1 in ESCC has not been examined. In the present study, we analyzed the expression and distribution of *KIFC1* in 132 ESCC cases by immunohistochemistry. In contrast to weak or no staining of KIFC1 in non-neoplastic mucosa, ESCC tissue showed stronger, more extensive KIFC1 staining. In total, 95 (72%) of 132 ESCC cases were positive for KIFC1. Immunostaining of ALDH1 was also performed, and KIFC1-positive ESCC cases were significantly frequently found in ALDH1-positive ESCC cases compared with ALDH1-negative ESCC cases. Spheroid colony formation is an effective method to characterize CSCs, thus we analyzed sphere number and size at 15 days in ESCC cells downregulated for KIFC1 by siRNA transfection. Both the number and size of sphere from TE-1 cells were significantly reduced in KIFC1 siRNA-transfected TE-1 cells than in negative control siRNA-transfected cells. These results suggest that KIFC1 plays an important role in ESCC pathogenesis.

1. Introduction

Esophageal cancer is the sixth most common malignancy worldwide [1]. The two predominant forms of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and adenocarcinoma. Globally, ESCC accounts for more than 90% of esophageal cancer. Most ESCC is diagnosed at an advanced stage, and even superficial ESCC that appears to extend no further than the submucosa metastasizes to the lymph nodes in 50% of cases [2]. For localized ESCC, surgery is the primary therapeutic option. However, the prognosis is unsatisfactory, even in curatively resected patients, and the 5-year survival rate is < 50% after surgery [3]. Several prognostic markers, such as nodal status and tumor stage, are currently accepted for clinical use, and we also previously identified several ESCC-associated genes [4–6]. However, these genes cannot completely identify which patients are at low or high risk for disease recurrence. Therefore, there is an urgent need for new prognostic markers and therapeutic targets for ESCC.

In the past decade, cancer has been recognized as a stem cell disease [7], and cancer stem cells (CSCs) have been described in numerous solid tumors. One effective method to characterize CSCs is spheroid colony formation [6,8]. We previously found upregulation of KIFC1 protein expression in 37% of gastric cancer cases [9]. We also found that the *KIFC1* gene was expressed more than two-fold higher in spheroid body-forming cells than in parental cells in a gastric cancer cell line and that both the number and size of spheres from gastric cancer cell lines were significantly reduced by knockdown of *KIFC1*. KIFC1 protein (also known as HSET) is a C-type kinesin of the kinesin-14 family [10] and is a minus end-directed motor protein [11]. Kinesins are a family of molecular motors that play important roles in intracellular transport or cell division [12]. Alteration of several kinds of kinesins have been reported in human cancers [12]. Upregulation of KIFC1 has been shown in human breast cancer [13], ovarian cancer [14], and lung cancer [15]. In breast cancer cells, forced expression of KIFC1 inhibits docetaxel-mediated apoptosis [16]. KIFC1 also induces resistance to docetaxel and is associated with survival of patients with prostate cancer [17]. These data support the idea that KIFC1 protein may be a potential novel tumor marker for a wide variety of malignancies. However, the expression of KIFC1 in ESCC has not previously

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been analyzed.

In the present study, we analyzed the expression and distribution of KIFC1 in human ESCC by immunohistochemistry and examined the relationship between KIFC1 expression and clinicopathologic characteristics. We also analyzed the effect of inhibiting KIFC1 expression by RNA interference (RNAi) on spheroid formation of ESCC cells.

2. Materials and methods

2.1. Tissue samples

In a retrospective study design, 132 primary tumors were collected from patients diagnosed with ESCC who underwent surgery between April 2003 and March 2008 at Hiroshima University Hospital (Hiroshima, Japan). All patient samples were obtained with consent, and the present study was approved by the Ethical 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 the patient 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 scan and serum chemistries were performed at every follow-up visit. Patients were followed by the patients' physician until the patient's death or the date of the last documented contact.

Archival formalin-fixed, paraffin-embedded tissues from 132 patients who had undergone surgical excision for ESCC were examined by immunohistochemical analysis. 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 the lateral and deep tumor invasive front. Tumor staging was determined according to the TNM classification system [18].

2.2. Immunohistochemistry

Immunohistochemical analysis was performed with the Dako Envision+ Mouse Peroxidase Detection System (Dako Cytomation, Carpinteria, CA, USA) as described previously [9]. 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 (Dako Cytomation) for 20 min to block nonspecific antibody binding sites. Sections were incubated with a mouse monoclonal anti-KIFC1 antibody (1:50, H00003833-M1, Abnova, Taipei, Taiwan) 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. The immunostaining of ALDH1 was performed as previously described [6].

Expression of KIFC1 was scored in all tumors as positive or negative.

Immunostaining for KIFC1 was considered positive when more than 10% of tumor cells were stained. Using these definitions, two surgical pathologists (NO and KS), 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.

2.3. Cell lines

Four cell lines derived from human esophageal cancer (TE-1, TE-5, TE-10, and TE-11) were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Cell line identity was verified by short tandem repeat profiling (Promega, Madison, MD, USA). All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.4. RNAi

RNAi was performed as described previously [9]. Short interfering RNA (siRNA) oligonucleotides targeting *KIFC1* and a negative control were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). We used two independent *KIFC1* siRNA oligonucleotide sequences. Transfection was performed using Lipofectamine RNAiMAX (Life Technologies) as described previously [19]. 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 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.

2.5. Western blot analysis

Western blot analysis was performed as described previously [20]. Protein concentrations were determined by Bradford Protein Assay (Bio-Rad, Richmond, CA, USA) with BSA used as the standard. 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 filter. Anti-KIFC1 antibody (1:500) was purchased from Abnova (H00003833-M1). Peroxidase-conjugated anti-mouse IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Life Technologies) according to the manufacturer's instructions. β-Actin (AC-15, Sigma Chemical, St. Louis, MO, USA) was also stained as a loading control.

2.6. Sphere formation assays

A total of 2000 cells were plated per well on 24-well ultra-low attachment plates (Corning, New York, NY, USA) 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 determined and counted under a microscope. Three independent experiments were carried out. Mean ± S.D. was calculated for each of the experiments.

2.7. Statistical methods

Associations between clinicopathologic 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. A *P* value of less than 0.05 was considered statistically significant. SPSS version 8.0 software was used for these analyses (SPSS, Chicago, IL, USA).