

Enhanced expression of KIF4A in colorectal cancer was associated with histological type and lymph node metastasis

Yoshiko Matsumoto

Department of Organ Regulatory Surgery

Fukushima Medical University

Abstract

Purpose: Colorectal cancer (CRC) is one of the most common and fatal cancers in the world. Recent molecular target treatment, including an anti-EGFR antibody, improves the overall survival of CRC patients with *RAS* wild-type. *KIF4A* is considered to be a component of the chromosome condensation and segregation machinery functioning in multiple steps of mitotic division. KIF4A expression was up-regulated in cervical, lung, oral, and breast cancer. The role of *KIF4A* in CRC is still unclear.

Methods: We first performed the comparison of *KIF4A* mRNA expression between cancer tissue and normal mucosa in 63 CRC cases by a realtime RT-PCR method. Next, KIF4A expression was evaluated by an immunohistochemical staining method in 258 CRC patients who underwent surgical operation at our institute, and analyzed the relationship between KIF4A expression and clinicopathological characteristics. We further investigated the biological significance of KIF4A expression in CRC using colorectal cancer cell lines.

Results: *KIF4A* mRNA expression was significantly up-regulated in cancer tissue when compared to normal mucosa ($p=0.002$). KIF4A expression was enhanced in both nucleus and cytoplasm of the cancerous tissue, while no KIF4A expression was observed in normal mucosa. Strong expression of KIF4A in cancer tissue was detected

in 132 cases (51.2%). Its expression was significantly associated with lymph node metastasis ($p<0.05$) and histological type ($p<0.05$). There was no association between KIF4A expression and prognosis in CRC patients. Knockdown of KIF4A expression using small interfering RNA oligos in a colon cancer cell line, HCT116, led to inhibition of cellular proliferation ($p<0.001$).

Conclusion: In the present study, we found that KIF4A expression was enhanced in approximately 50% of CRC patients and was associated with lymph node metastasis and histological type. Enhanced expression of KIF4A in colorectal cancer might be associated with cancer cellular proliferation.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in Japan. Recent molecular target treatment, including an anti-EGFR antibody, improves the overall survival of CRC patients with *RAS* wild-type (1). However, responders for chemotherapy are still limited up to 50%. Numerous investigators have focused on screening for novel diagnostic and prognostic biomarkers as well as therapeutic targets in CRC.

The kinesin superfamily proteins (KIFs) are microtubule-dependent molecular motors that convert the chemical energy of ATP hydrolysis to the mechanical force of transporting cargos along microtubules, suggesting that play important roles in intracellular transport and cell division (2). They are classified into 14 distinct families with varying structural and functional characteristics (3, 4). Among the KIFs, *KIF4A* is considered to be a component of the chromosome condensation and segregation machinery functioning in multiple steps of mitotic division (5, 6). Dysregulation of *KIF4A* induces abnormal spindle separation and causes aneuploidy of daughter cells. Cells affected by aneuploidy are characterized by gain or loss of genetic material. Therefore, *KIF4A* expression is considered to be associated with cancer progression.

It has been reported that kinesin *KIF4A* expression is altered in various types of

cancers, including cervical (7), lung (8), gastric (9), oral (10), and breast cancer (11). These alterations in cancer cells imply the biological function of KIF4A that are associated with regulation of the cell cycle and cellular proliferation. A previous microarray study disclosed the elevated expression of KIF4A mRNA in human cervical cancer (7). KIF4A expression was up-regulated in lung cancer and was significantly associated with male gender, nonadenocarcinoma histology, and shorter survival in non-small cell lung cancer patients (8). In immunohistochemical evaluation of 106 oral squamous cell cancer patients, KIF4A expression of cancer tissue was significantly stronger than that of normal tissue (10). A recent report has shown that estrogen induced many KIFs including KIF4A, and elevated level of KIF4A correlated with poor relapse free survival of breast patients with positive estrogen receptor (11). These results indicated that KIF4A might function as an oncogene. On the other hand, KIF4A have the opposite effect as a tumor suppressor gene in gastric cancer (9).

In this study, we investigated KIF4A mRNA and protein expression in CRC and its biological significance. We here showed that KIF4A expression was enhanced in a half of CRC, and enhanced expression of KIF4A might be associated with cellular proliferation in CRC.

Material and Methods

Tissue samples

A total of 258 surgical specimens obtained from the CRC patients who underwent surgical resection in the Fukushima Medical University Hospital between January 1991 and December 2011, were used for experiments. Of 258 specimens, mRNA was extracted from both cancer tissue and normal mucosa in 63 cases. Malignant and adjacent nonmalignant portions of each specimen were used for RNA extraction. The clinical characteristics were shown in Table 1. The histological diagnosis of the resected specimen was determined according to the Japanese classification of colorectal carcinoma (12, 13). The carcinomas at the time of primary tumor resection were staged according to the UICC classification. This study was performed in accordance with the Ethical Guidelines for Clinical Research with approval of the Institutional Ethics Committee. Informed consent was obtained from the individuals included in the study.

Cells and Cell culture

Human cultured colorectal cancer cell lines, HCT15, HCT116 (14), LST174T, LS180, LoVo, RKO, SW48, SW480, and SW620, were originally obtained from the American Type Culture Collection (Rockville, MD, USA). These cell lines were grown at 37 °C under the presence of 5 % CO₂ in the recommended culture media with 10 % fetal

bovine serum (NICHIREI BIOSCIENCES INC., JAPAN) .

RNA Extraction and Quantitative Realtime Reverse transcription-Polymerase Chain Reaction (realtime RT-PCR)

Extraction of total RNA from CRC specimens and CRC cell lines was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA (5 µg) of each sample was used for synthesis of cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) following the manufacture's instruction. The level of *KIF4A* mRNA expression was evaluated using ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA) with TaqMan probes provided by manufacturer. TaqMan Probes of KIF4A (Hs01020169_m1) and β -actin (Hs99999903-m1) were obtained from Applied Biosystems and used for RT-PCR analysis. The relative amount of β -actin transcripts used as the internal control in the same sample, and described as the ratio of *KIF4A*/ β -actin.

The PCR condition was as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 30 sec, and 72 °C for 7 min, then reserved 4 °C. Using the comparative threshold cycle ($\Delta\Delta C_t$) method, the relative expression of the target genes was normalized to the endogenous β -actin.

Immunohistochemistry (IHC) and IHC Evaluation

Immunohistochemical staining was carried out on paraffin-embedded histological sections (4 μ m thick) using a polymer peroxidase method. Briefly, after deparaffinization and rehydration, sections were treated with 0.3 % hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After a rinsing in PBS, the sections were incubated with anti-KIF4A antibody (rabbit polyclonal antibody (ab122227), 1:300 dilution; Abcam) and anti-MIB-1 antibody (mouse monoclonal antibody, 1:100 dilution; Dako) at 4 °C overnight. A further wash in PBS was followed by treatment with peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulins (ENvision+kit; Dako Cytomation, Glostrup, Denmark) as the secondary antibody for 30 min at room temperature. The staining was visualized with diaminobenzidine (DAB), followed by counterstaining with hematoxylin. Expression of these proteins was evaluated as positive when the nucleus of cancerous tissue when the total field of view was observed at 40 \times magnification. The staining of each specimen was evaluated by us blinded to origination of the features and clinical outcomes. Staining cancer cells were counted per 1000 cancer cells in the maximum field of cancer tissue by two investigators. Positive rate was classified as follows: 0-5%; 0, 6-20%; 1, 21-50%; 2, 51-100%; 3. The staining intensity was scored as 0 (negative), 1 (weak), 2

(moderate), 3 (strong). The evaluation was expressed as a product of the score of positive rate and staining intensity. Positive staining was defined with more than 2, while negative staining was scored at 0 or 1.

Protein Extraction and Western blot

Cells were washed twice in ice-cold DPBS (Invitrogen), pelletized by centrifugation method (1500 rpm 5 min), and stored in -80 °C freezer. Pellet was dissolved in RIPA Buffer (Thermo SCIENTIFIC) with Halt Protease Inhibitor Single-Use Cocktail (100×) (Thermo SCIENTIFIC), and centrifuged at 4 °C, 15000 rpm for 20 min. Total protein concentration was measured by a Bradford method using Bradford reagent (BIO RAD) and Smart Spec 3000 (BIO RAD). Total protein isolated from cell lines was separated by SDS-PAGE. Tris-Glycine SDS sample buffer (Invitrogen, USA) and 3-Mercapto-1, 2-propanediol (Wako, Japan) were added into the protein and samples were heated at 100 °C for 3 min. The 4-12% Tris-Glycine gels (Life technology) filling with proteins electrophoresed in 100 V for 100 min, using Tris-Glycine SDS Running Buffer (Life technology) in X Cell SureLock (Life technology). After electrophoresed, the gel was transferred onto the nitrocellulose membrane (iBlot Gel Transfer Stacks Nitrocellulose Mini (Life technology)), blocked in SuperBlock Blocking Buffer in PBS (Thermo

SCIENTIFIC). The protein blots were incubated with an anti-KIF4A antibody (rabbit monoclonal antibody, abcam) for 1 hour at room temperature, and then incubated with a goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) as a second antibody for 30 min at room temperature. Bound antibodies were detected by enhanced chemiluminescence detection reagents (Thermo SCIENTIFIC) and visualized by autoradiography (LAS-4000 IR MultiColor) (FUJI, Tokyo, Japan). The protein levels were quantified using a rabbit monoclonal anti- β -actin antibody (Santa Cruz Biotechnology) as the internal loading control.

siRNA transfection

Knockdown experiment was performed by a small interfering RNA (siRNA) method using KIF4A siRNA oligos (siRNA (h): sc-60888, Santa Cruz Biotechnology) and Lipofectamine RNA-iMAX (Life Technology) according to the manufacturer's protocol. One day before transfection, a CRC cell line, HCT116, was seeded 15×10^5 cells per well in 6-well plate. Transfection with the final concentration of 10 nM siRNA was conducted when the cell density was approximately 30-50 % in 6-well plates, and incubated 48 hours.

Cell Counting

The Cell Counting kit-8(DOJINDO JAPAN) was used to measure the cell viability. HCT116 cells were seeded at the density of 2000 per well in 96-well microplates with 100µl RPMI1640 and incubated overnight. Then, transfection with the final concentration of 100 pmol siRNA was conducted and incubated 5 days. Every day after transfection, 10 µl CCK-8 solution was added into cells at same time and incubated 2 hours. The absorbance value at 450nm was measured using fluorescence microplate reader (Bio-Rad Laboratories; Benchmark Plus, USA).

Invasion assay

An in vitro fluoroblok tumor invasion assay was purchased from BD Biosciences (#354165). CRC cells, HCT116, were seeded and cultured until subconfluence. Each 500 µl of cell suspension (2.5×10^4 cells) was added to the apical chambers, and 750 µl of chemoattractant (5% FBS in RPMI1640) to each of the basal chambers. The BD BioCoat Tumor Invasion System and the uncoated BD Falcon FluoroBlok 24-Multiwell Insert Plate were incubated at 37°C for 20-22 hours. Following incubation, the medium was carefully removed from the apical chambers. The insert system was transferred into a second 24-well plate containing 500 µl/well of 4 µg/ml Calcein AM in HBSS and

incubated for 1 hour. Fluorescence of invaded cells is read at wavelengths of 494/517 nm (Ex/Em) on a bottom-reading fluorescent plate reader.

Statistical Analysis

Statistical analyses were carried out by StatMate V software (ATMS, JAPAN). Continuous variables were applied using the independent t-test and χ^2 test for the analysis of relationship between KIF4A expression and clinicopathological characteristics. Survival curves were generated by the Kaplan-Meier method and compared by log-rank test. The difference was considered to be statistically significant at $p < 0.05$.

Results

KIF4A mRNA expression was up-regulated in colorectal cancer.

Of 63 CRC patients, *KIF4A* mRNA expression of cancer tissue was two times higher than that of normal tissue in 32 CRC patients. The median ratio to β -actin of *KIF4A* mRNA in cancer tissue and normal tissue was 3.55 and 2.07, respectively (Table 2). Totally, *KIF4A* mRNA expression was significantly up-regulated in colorectal cancer ($p=0.002$) (Fig.1).

KIF4A expression and clinical significance in CRC patients

Of 258 colorectal cancer patients, high level of KIF4A expression was detected in 132 (51.1%) patients by an immunohistochemical evaluation. KIF4A expression was observed in nucleus and cytoplasm of cancer cells, while no expression was observed in normal mucosa (Fig. 2). The analysis of the relationship between KIF4A expression and clinicopathological characteristics was shown in Table 2. KIF4A expression was significantly associated with histology ($p < 0.05$) and lymph node metastasis ($p < 0.05$). Significant correlations between KIF4A expressions and other factors, including gender, age, tumor location, stages, depth of invasion, lymphatic invasion, venous invasion, or liver metastasis were not observed. The overall survivals (OS) did not differ between patients with high KIF4A expression and those with low KIF4A expression (Fig. 3).

Knock down of KIF4A induces cellular growth inhibition

Of 9 human colorectal cancer cell lines, *KIF4A* mRNA expression of HCT116 cells was relatively higher than that of other cell lines (Fig. 4A). The level of KIF4A protein expression was also high in HCT116 cells (Fig. 4B). Therefore, HCT116 cells were used for siRNA experiments.

First, the effect of KIF4A siRNA was validated. The level of KIF4A mRNA and

protein was significantly decreased when KIF4a siRNA oligos were transfected into HCT116 cells, while no change was observed in control cells (Fig. 5A and 5B). A morphological change was not observed in all of the knocked-down KIF4A cells. Significant suppression of cellular proliferation was observed in HCT116 cells treated with KIF4A siRNA compared to those treated with control siRNA ($p<0.05$) (Fig. 6). But, no significant change of cellular invasive activity was observed in HCT116 cells treated with KIF4A siRNA compared to those treated with control siRNA ($p=0.81$) (Fig. 7).

The relationship between histological type and KIF4A expression and MIB-1 Labeling Index in colorectal cancer

To verify the hypothesis that higher KIF4A expression was associated with differentiation in the histological type, we first examined the relationship between histological type and MIB-1 Labeling Index (MIB-1 LI) in 45 colorectal cancer cases, including 15 well-differentiated adenocarcinoma, 15 moderately-differentiated adenocarcinoma, and 15 poorly-differentiated and others. MIB-1 LI of each histological type was $69.4\pm14.2\%$, $66.6\pm12.9\%$, and $71.8\pm14.0\%$, respectively (Fig. 8). There was no relationship between histological type and MIB-1 LI ($p=0.71$).

Next, the staining score of KIF4A expression was compared to MIB1 LI in 45

colorectal cancer cases. No correlation was observed among these expressions ($p=0.39$) (Fig. 9).

Discussion

In this study, we found that KIF4A expression was enhanced in approximately 50% cases in CRC. Enhanced KIF4A expression was associated with histological type and lymph node metastasis in CRC. Suppression of cellular proliferation was found in KIF4A knocked-down cells, leading to suggesting that enhanced KIF4A expression might be associated with cellular proliferation in CRC.

Previous studies have showed that KIF4A expression is constantly up-regulated in proliferating cultured cancer cells and various types of cancer, including cervical (7), lung (8), oral (10), and breast cancer (11). Regarding clinical significance in lung cancer, up-regulated KIF4A expression was significantly associated with male gender, nonadenocarcinoma histology, and shorter survival in non-small cell lung cancer patients (8). In immunohistochemical evaluation of 106 oral squamous cell cancer patients, KIF4A expression of cancer tissue was significantly stronger than that of normal tissue and was associated with tumor size (10). In the present study, we showed that enhanced KIF4A expression in colorectal cancer was significantly associated with

histological type and lymph node metastasis, while survival outcome had no significant difference between CRC patients with positive KIF4A expression and negative KIF4A expression. These results indicated that KIF4A expression might be associated with cellular differentiation and progression.

Other reports indicated that KIF4A functions as a tumor suppressor gene (9, 15). KIF4 KO mouse ES cells have mitotic defects and aberrant chromosome structure and are aneuploid (15). They suggested that loss of KIF4 increases the potential of ES cells for tumor formation (15). In addition, loss of KIF4A could onset carcinogenesis as a subset of cancer cell lines they tested among the NCI-60 tumor cell line collection express loss or weak KIF4A, while no reduction of KIF4 was found in colon cancer cell lines (15). The analysis of 23 gastric carcinoma specimens, 13 cases (56.6%) had lower expression of KIF4 compared with corresponding adjacent normal tissues (9). There was a significant correlation between low expression of KIF4 and poor differentiation of tumor. They suggested that KIF4A functions as an inhibitor of gastric cancer cell proliferation based on biological experiments. These results were opposite findings against our and other results (7, 8, 10, 11). However, these phenomena appear to be similar. These results suggest that a physiological range of KIF4A levels is important to maintain cellular homeostasis. Further studies with either KIF4A transgenic or knockout

mice will be interesting to pursue.

We then examined to identify biological significance of up-regulated KIF4A expression in CRC. Knockdown of KIF4A expression in a colon cancer cell line, HCT116, induced suppression of cellular proliferation. A previous report (5) demonstrated that KIF4A knockout (KO) cells were constructed using HCT116 cells as we used. The result showed that HCT116 KIF4A KO cells proliferated slower than wild-type cells and mitosis was slightly prolonged in HCT116 KIF4A KO cells. These findings support our results that up-regulated KIF4A expression in CRC was associated with cellular proliferation. In lung cancer, knockdown of KIF4A expression suppressed cellular proliferation using a lung squamous cell carcinoma cell line, SBC-5 (8). Moreover, KIF4A was linked to regulation of cell cycle in the M phase and controls cellular proliferation via activation of the spindle assembly checkpoint in oral squamous cell carcinomas (OSCCs) (10). Down-regulation of KIF4A induced the cell arrest of OSCC cells by similar functions of the microtubule inhibitors. Therefore, enhanced KIF4A expression in cancer cells is considered to be associated with cellular proliferation.

In vitro experiments suggested that alteration of KIF4A expression was associated with cellular proliferation in colorectal cancer. We then investigated the correlation

between KIF4A and MIB-1 LI in colorectal cancer specimens. In this study, positive KIF4A expression was associated with higher differentiation of histological type in clinicopathological characteristics. Although it has been known that the cellular proliferation is inversely associated with cellular differentiation, no correlation was found in MIB-1 LI and each histological types. Moreover, there was no relationship between KIF4A expression and MIB-1 LI in colorectal cancer specimens.

To the best of our knowledge, our finding that KIF4A expression was enhanced in approximately 50% cases in CRC is the first report. Moreover, we demonstrated that up-regulated KIF4A expression was associated with histological type and lymph node metastasis. Since knockdown of KIF4A successfully suppressed cellular proliferation, KIF4A might apply to a molecularly-targeted drug like an anti-EGFR antibody, leading to improvement of the overall survival of CRC patients.

References

1. Heinemann V, von Weikersthal LF, Decker T, Kiani A, Vehling-Kaiser U, Al-Batran SE, Heintges T, Lerchenmüller C, Kahl C, Seipelt G, Kullmann F, Stauch M, Scheithauer W, Hielscher J, Scholz M, Müller S, Link H, Niederle N, Rost A, Höffkes HG, Moehler M, Lindig RU, Modest DP, Rossius L, Kirchner T, Jung A, Stintzing S. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer (FIRE-3): a randomised, open-label, phase 3 trial. *Lancet Oncol.* 2014 Sep;15(10):1065-75.
2. Nakagawa T, Tanaka Y, Matsuoka E, Kondo S, Okada Y, Noda Y, Kanai Y, Hirokawa N. Identification and classification of 16 new kinesin superfamily (KIF) proteins in mouse genome. *Proc Natl Acad Sci U S A.* 1997 Sep 2;94(18):9654-9.
3. Lawrence CJ, Dawe RK, Christie KR, Cleveland DW, Dawson SC, Endow SA, Goldstein LS, Goodson HV, Hirokawa N, Howard J, Malmberg RL, McIntosh JR, Miki H, Mitchison TJ, Okada Y, Reddy AS, Saxton WM, Schliwa M, Scholey JM, Vale RD, Walczak CE, Wordeman L. A standardized kinesin nomenclature. *J Cell Biol.* 2004 Oct 11;167(1):19-22.]
4. Miki H, Okada Y, Hirokawa N. Analysis of the kinesin superfamily: insights into structure and function. *Trends Cell Biol.* 2005 Sep;15(9):467-76.

5. Wandke C, Barisic M, Sigl R, Rauch V, Wolf F, Amaro AC, Tan CH, Pereira AJ, Kutay U, Maiato H, Meraldi P, Geley S. Human chromokinesins promote chromosome congression and spindle microtubule dynamics during mitosis. *J Cell Biol.* 2012 Sep 3;198(5):847-63.
6. Mazumdar M, Sundareshan S, Misteli T. Human chromokinesin KIF4A functions in chromosome condensation and segregation. *J Cell Biol.* 2004 Aug 30;166(5):613-20.
7. Narayan G, Bourdon V, Chaganti S, Arias-Pulido H, Nandula SV, Rao PH, Gissmann L, Dürst M, Schneider A, Pothuri B, Mansukhani M, Basso K, Chaganti RS, Murty VV. Gene dosage alterations revealed by cDNA microarray analysis in cervical cancer: identification of candidate amplified and overexpressed genes. *Genes Chromosomes Cancer.* 2007 Apr;46(4):373-84.
8. Taniwaki M, Takano A, Ishikawa N, Yasui W, Inai K, Nishimura H, Tsuchiya E, Kohno N, Nakamura Y, Daigo Y. Activation of KIF4A as a prognostic biomarker and therapeutic target for lung cancer. *Clin Cancer Res.* 2007 Nov 15;13(22 Pt1):6624-31.
9. Gao J, Sai N, Wang C, Sheng X, Shao Q, Zhou C, Shi Y, Sun S, Qu X, Zhu C. Overexpression of chromokinesin KIF4 inhibits proliferation of human gastric

- carcinoma cells both in vitro and in vivo. *Tumour Biol.* 2011 Feb;32(1):53-61.
10. Minakawa Y, Kasamatsu A, Koike H, Higo M, Nakashima D, Kouzu Y, Sakamoto Y, Ogawara K, Shiiba M, Tanzawa H, Uzawa K. Kinesin family member 4A: a potential predictor for progression of human oral cancer. *PLoS One.* 2013 Dec 30;8(12):e85951
 11. Zou JX, Duan Z, Wang J, Sokolov A, Xu J, Chen CZ, Li JJ, Chen HW. Kinesin family deregulation coordinated by bromodomain protein ANCCA and histone methyltransferase MLL for breast cancer cell growth, survival, and tamoxifen resistance. *Mol Cancer Res.* 2014 Apr;12(4):539-49.
 12. Japanese Society for Cancer of the Colon and Rectum (2009) Japanese classification of colorectal carcinoma. Second English Edition. Tokyo: Kanehara & Co Ltd
 13. Takeuchi Y, Iishi H, Tanaka S, et al. Factors associated with technical difficulties and adverse events of colorectal endoscopic submucosal dissection: retrospective exploratory factor analysis of a multicenter prospective cohort. *Int J Colorectal Dis.* 2014 Oct;29(10):1275-84.
 14. Brattain MG, Fine WD, Khaled FM, Thompson J, Brattain DE. Heterogeneity of malignant cells from a human colonic carcinoma. *Cancer Res.* 1981

May;41(5):1751-6.

15. Mazumdar M, Lee JH, Sengupta K, Ried T, Rane S, Misteli T. Tumor formation via loss of a molecular motor protein. *Curr Biol.* 2006 Aug 8;16(15):1559-64.

Figure legend

Figure 1: *KIF4A* mRNA expression in colon cancer. Results of real-time RT-PCR analyses of mRNA levels of *KIF4A* in 63 human colon cancer tissues and normal mucosa. Paired *t* test was performed to ascertain statistical significance between the amount in cancer tissue and in normal mucosa. Y-axis shows the relative expression of β -actin as an internal control.

Figure 2: Expression of KIF4A in colorectal cancer tissues. Typical staining of KIF4A in colorectal cancer. Upper panel: cancer tissue, Lower panel: normal mucosa.

Figure 3: Kaplan-Meier curves of overall survival in patients with KIF4A positive expression and negative expression in colorectal cancer.

Figure 4: KIF4A expression in colorectal cancer cell lines. **A**, Results of real-time RT-PCR analyses of mRNA levels of *KIF4A* in colorectal cancer cell lines. Y-axis shows the relative expression of β -actin as an internal control. **B**, Western blot analysis of KIF4A expression in colorectal cancer cell line. Cell lysates from each cell lines were examined in western blot using anti- KIF4A antibody. β -actin was a loading control.

Figure 5: Validation of knock down effect of the KIF4A siRNA. **A**, HCT116 cells were transfected with the 40nM KIF4A siRNA (siRNA-KIF4A) or the 40 nM control siRNA (siRNA-control), and incubated for 48 hours. *KIF4A* mRNA expression was examined by Realtime RT-PCR. *KIF4A* expression levels were normalized by β -actin mRNA expression levels. **B**, Western blotting was performed for detecting KIF4A using the same samples as above. β -actin was probed as an internal control.

Figure 6: Effect of the KIF4A siRNA on cell growth. HCT116 cells were transfected with the 40nM siRNA- KIF4A or the 40nM siRNA-control, and incubated for 48 hours. The analysis was performed using Cell Counting Kit-8.

Figure 7: Effect of the KIF4A siRNA on tumor invasion. Using the HCT116 cells with siRNA-KIF4A, a cell invasion assay was performed using a 24-well BD BioCoat Tumor Invasion System as described in Material and Methods. Statistical analysis was performed by Student's *t* test. *Columns*, average of three independent experiments; *Bars*, SD.

Figure 8: The relationship between histological type and MIB-1 Labeling Index in colorectal cancer. The relationship between histological type and MIB-1 Labeling Index (MIB-1 LI) was investigated in 45 colorectal cancer cases, including 15 well-differentiated adenocarcinoma, 15 moderately-differentiated adenocarcinoma, and 15 poorly-differentiated and others. *Columns*, average of three fields of cancer tissue; *Bars*, SD.

Figure 9: Correlation between KIF4A and MIB-1 LI expression in colorectal cancer tissues. Statistical analysis was performed by Pearson's Correlation Coefficient.

Fig. 1

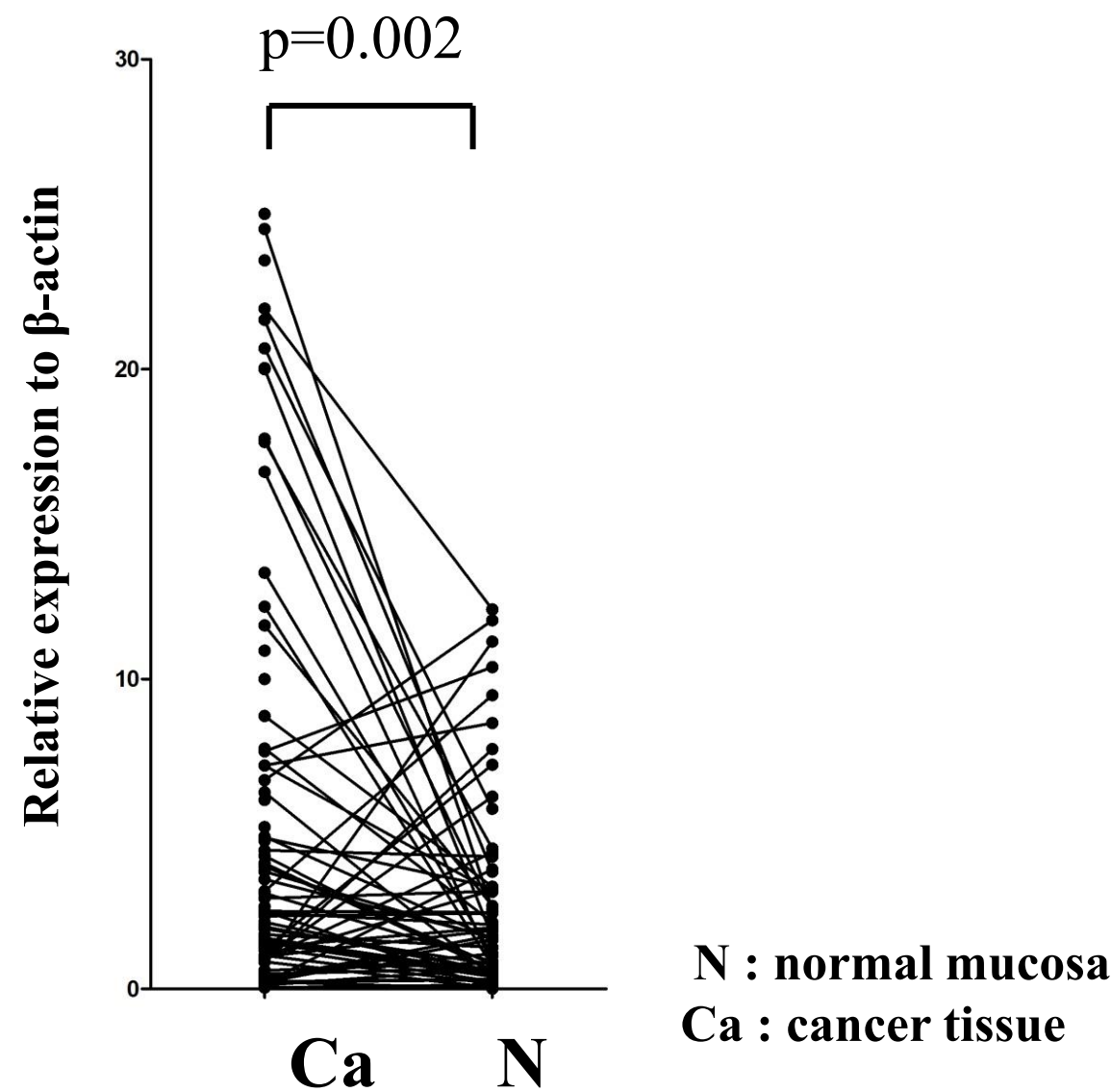


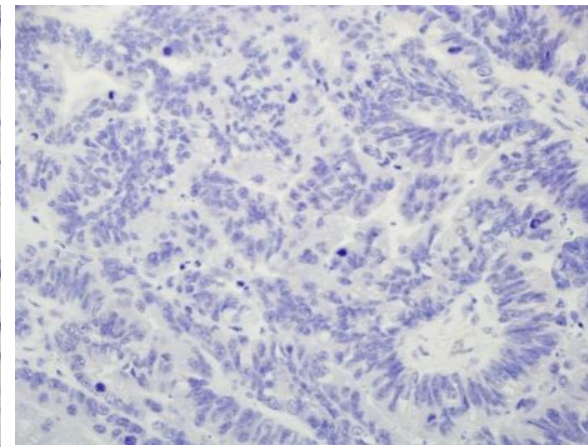
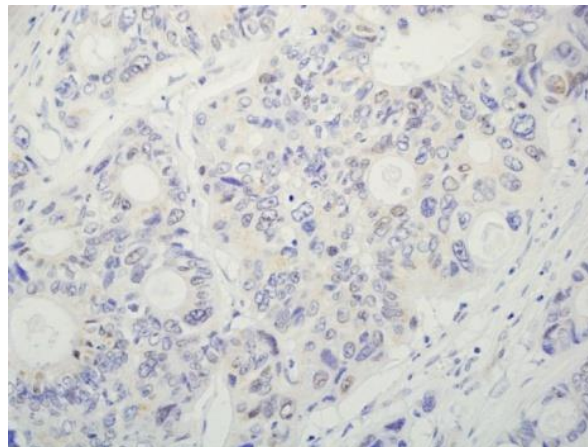
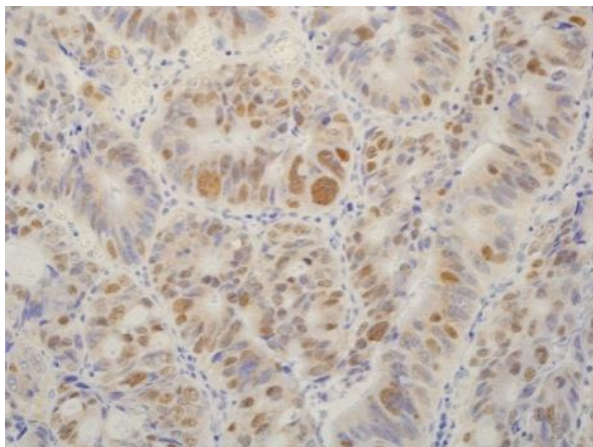
Fig. 2

**Strong
expression**

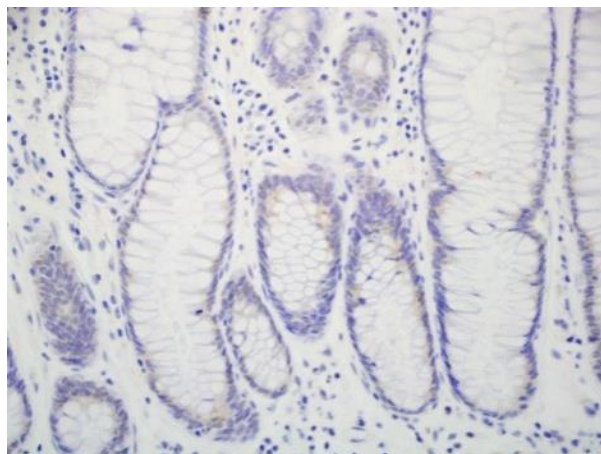
**Weak
expression**

**Negative
expression**

**Cancer
tissue**



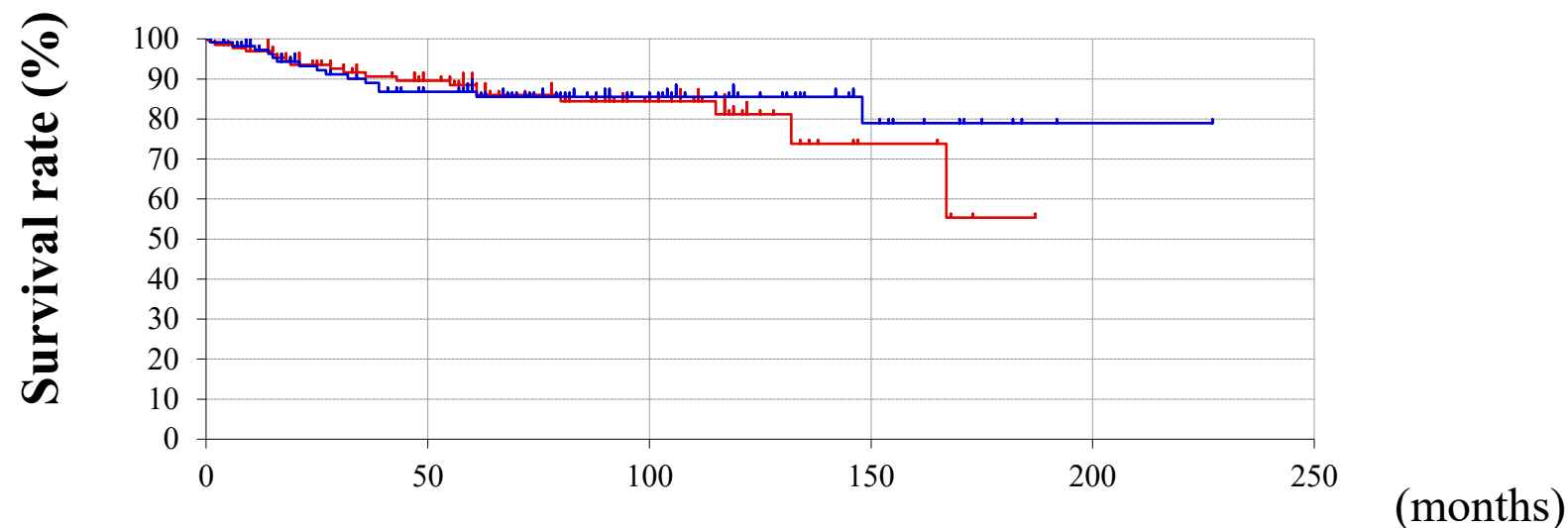
**Normal
tissue**



100μm



Fig. 3



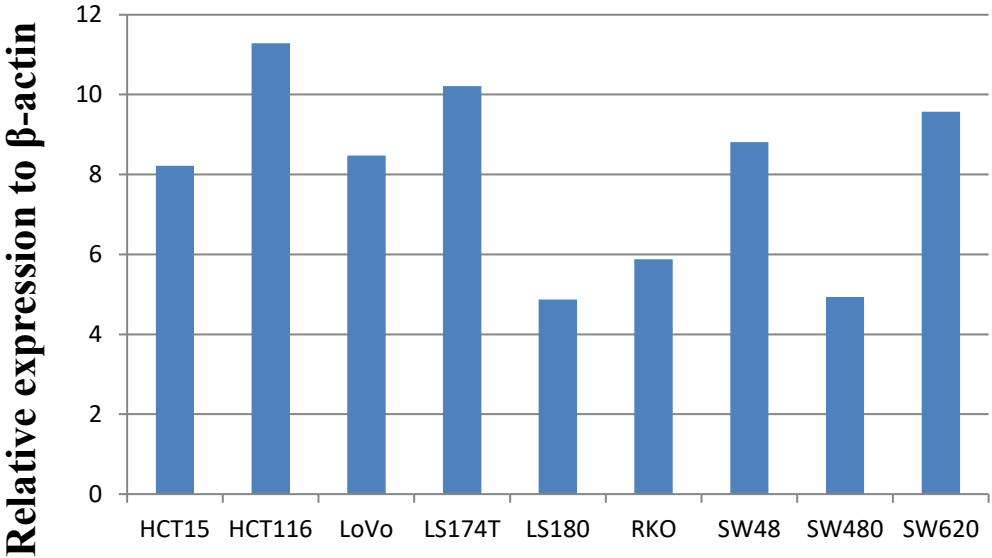
Duration of survival

KIF4A Positive ———
KIF4A Negative ———

Follow-up period: 1-230.8month
Median of follow-up period: 115.4months
Log –Rank test p=0.56

Fig. 4

A) mRNA expression



B) Protein expression

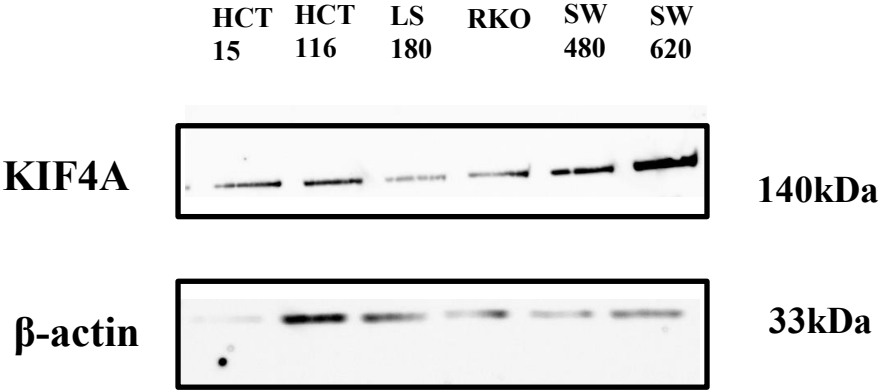
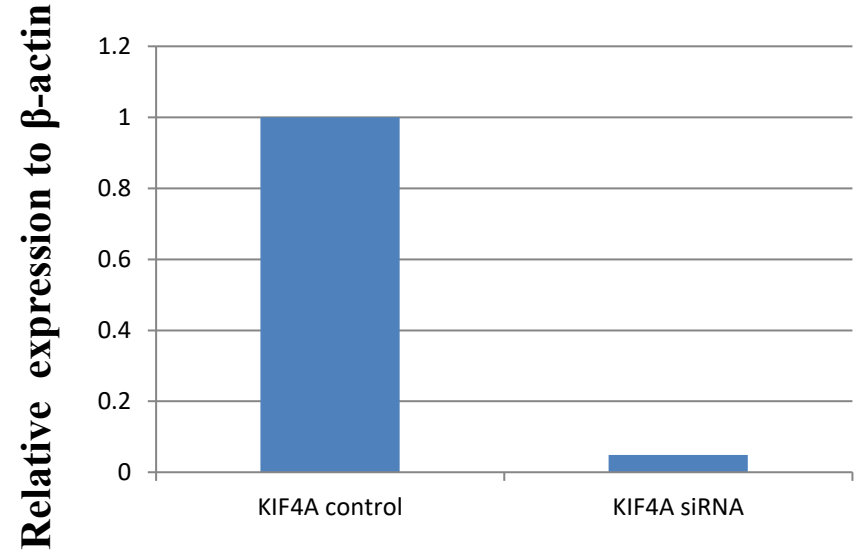


Fig. 5

A) mRNA expression



B) Protein expression

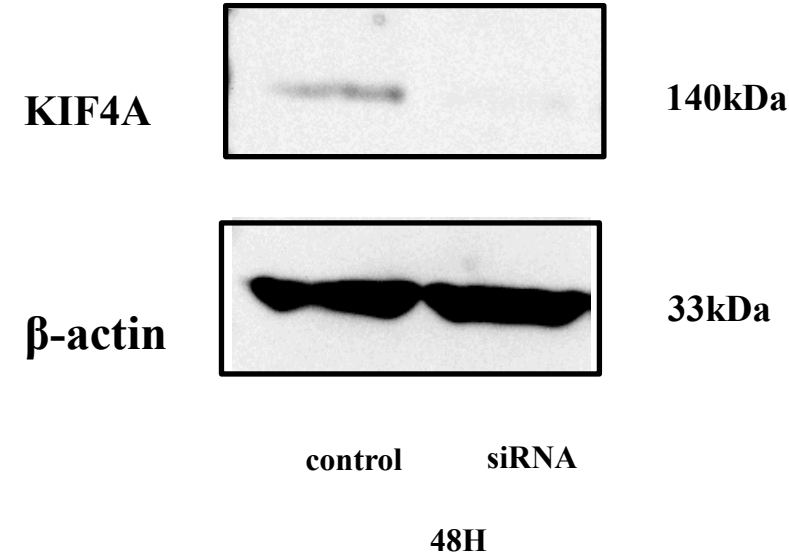


Fig. 6

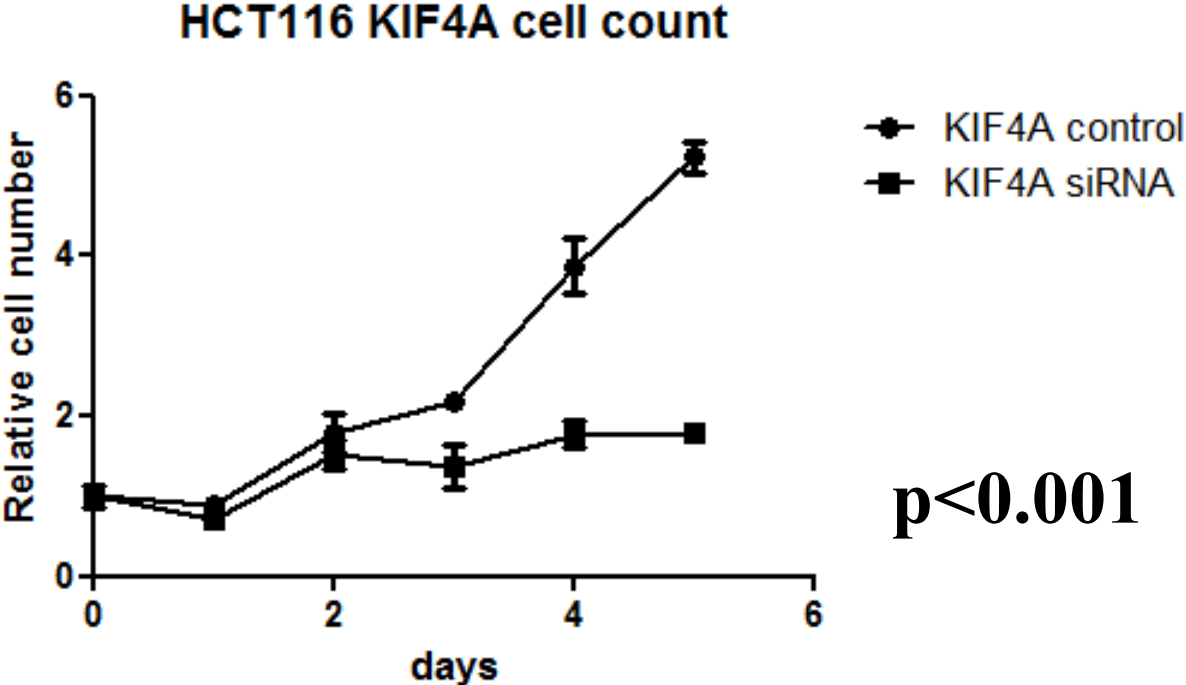


Fig. 7

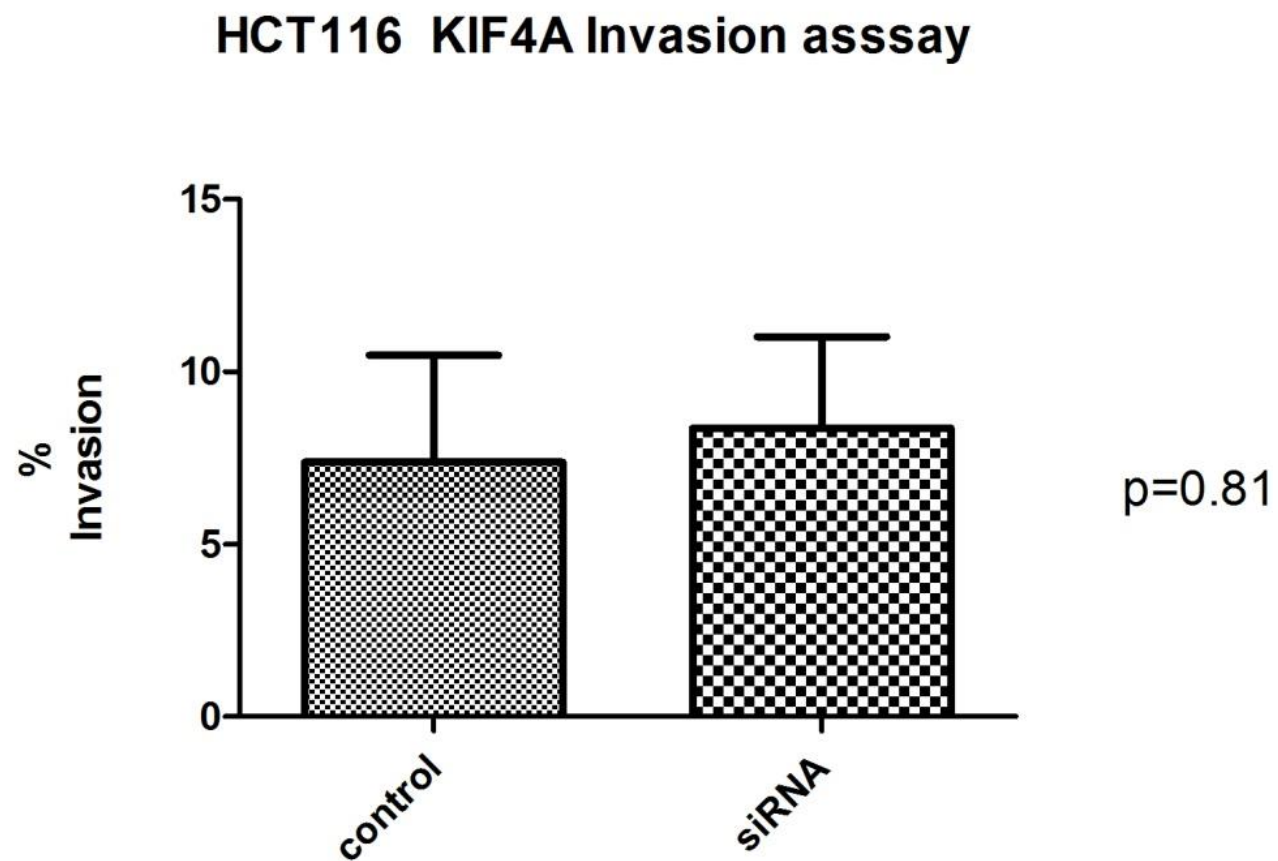


Fig. 8

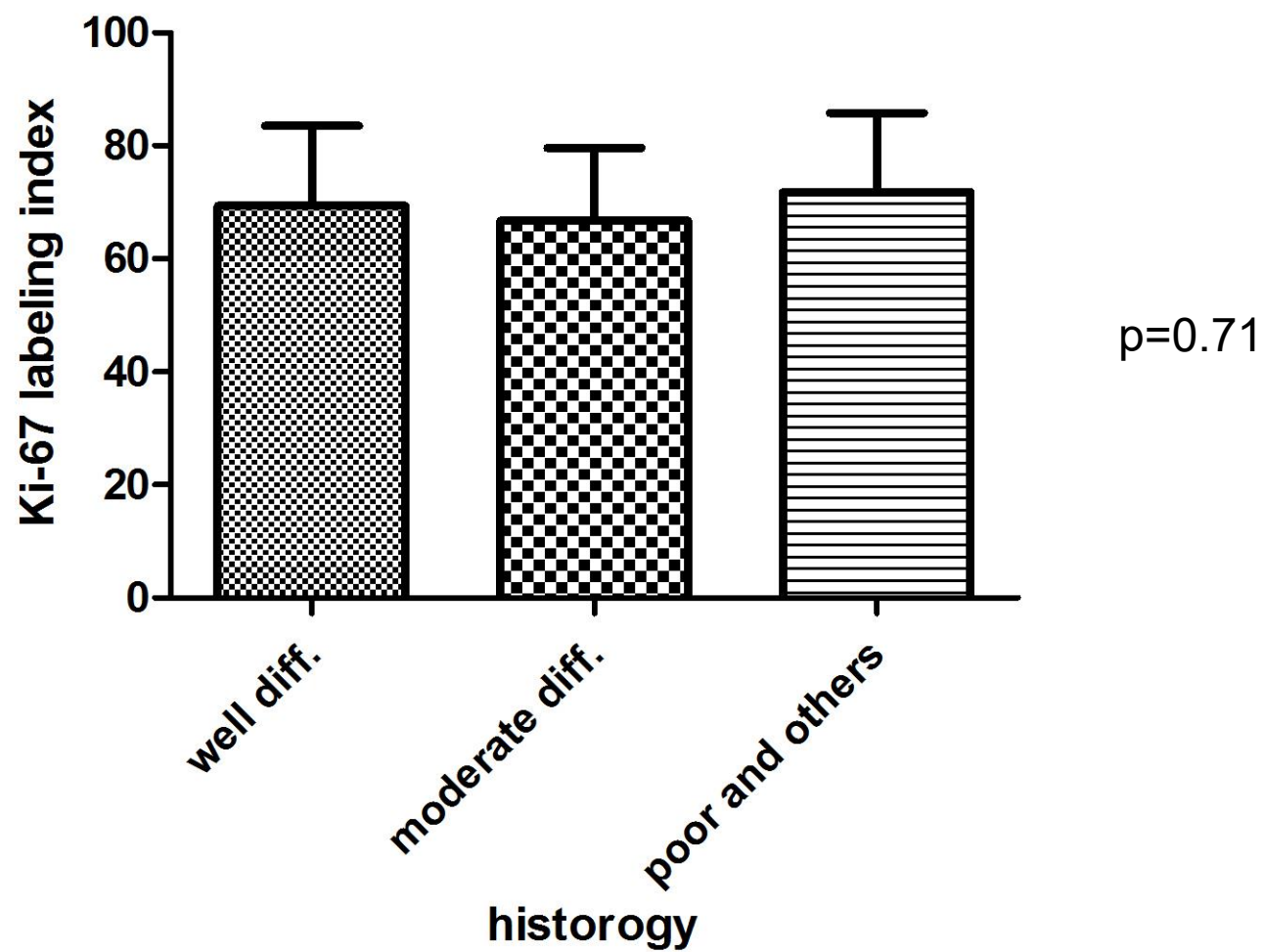


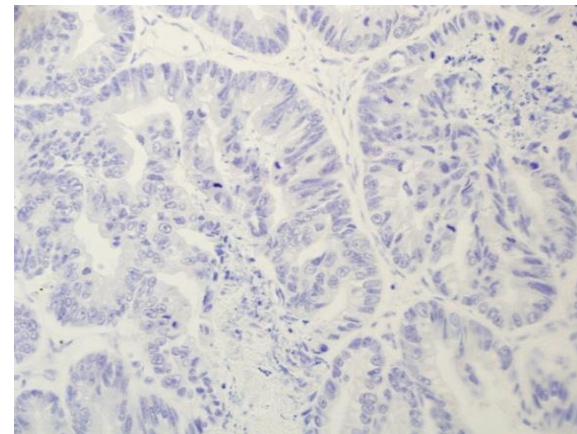
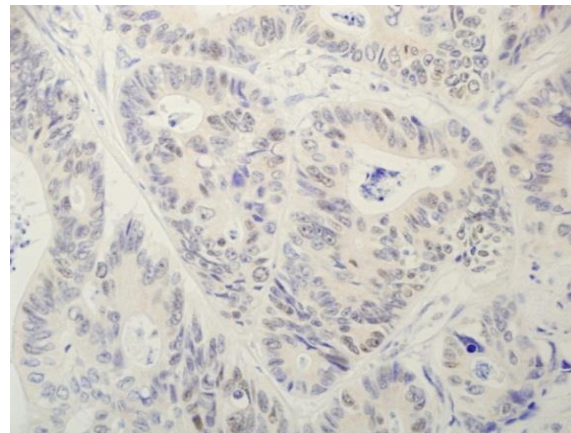
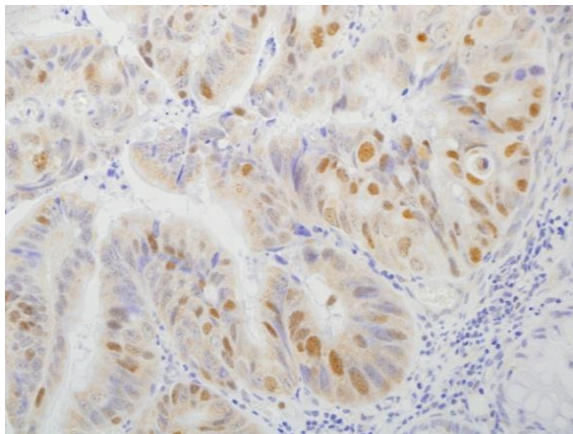
Fig. 9

**Strong
expression**

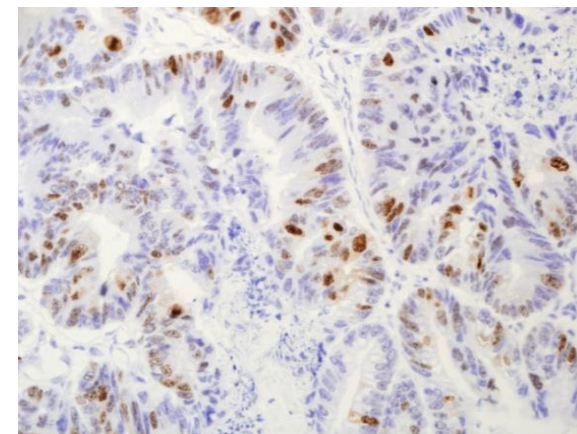
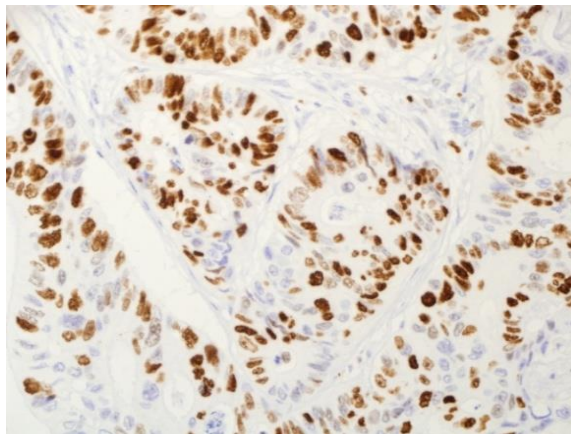
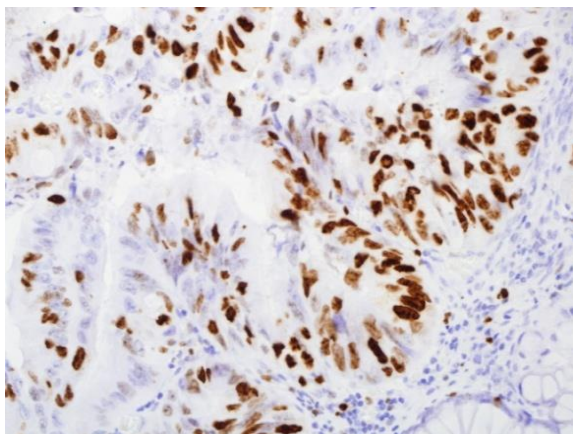
**Weak
expression**

**Negative
expression**

KIF4A



Ki-67



100μm

Fig.9

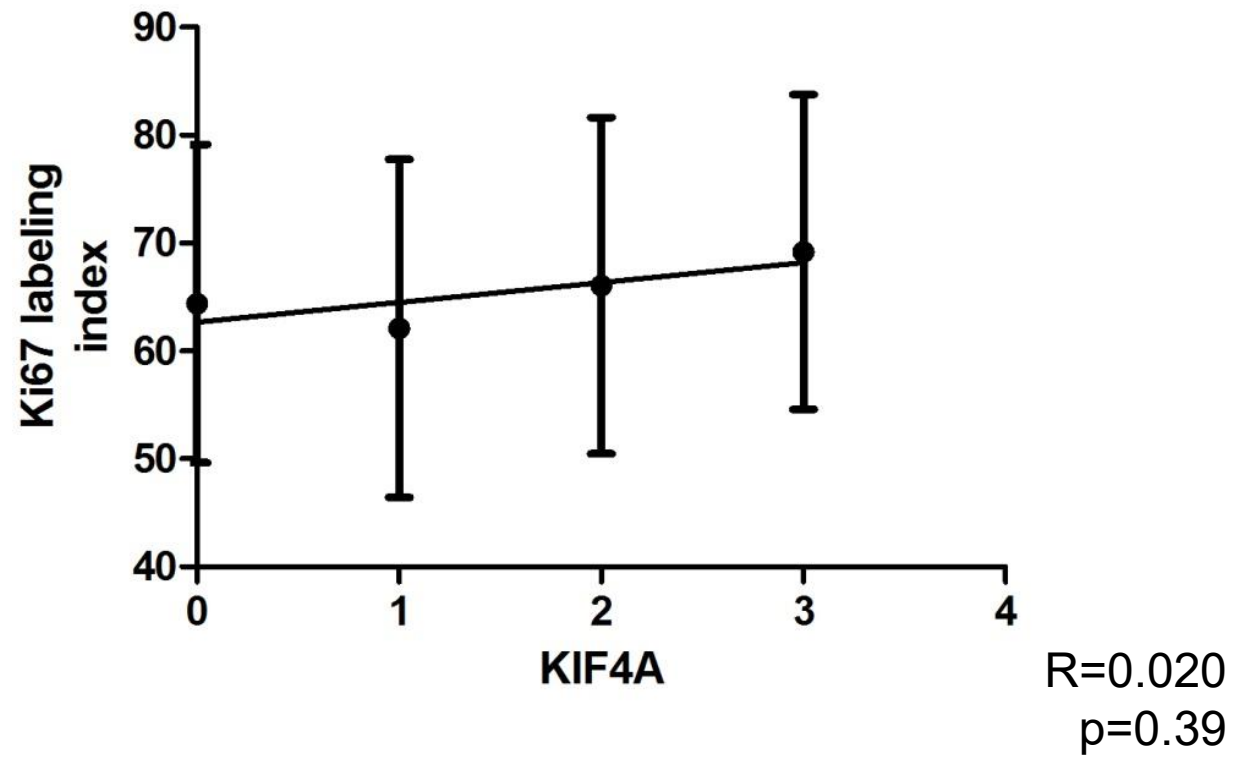


Table 1 The association between KIF4A expression and clinicopathological factors in 258 colorectal cancer patients

Factor		Total (n=258)	KIF4A		P-value
			Positive (n=132)	Negative (n=126)	
Gender	Male	149	79	70	0.49
	Female	109	53	56	
Age	<65	116	57	59	0.64
	≥65	142	75	69	
Stage	0	10	2	8	0.12
	I	40	23	17	
	II	93	43	50	
	III	75	48	27	
	IV	40	16	24	
Depth	Tis	10	2	8	0.22
	T1	26	13	13	
	T2	32	22	10	
	T3	172	95	82	
	T4	18	5	13	

Factor		Total (n=258)	KIF4A		P-value
			Positive (n=132)	Negative (n=126)	
Lymph node metastasis	Positive	95	59	36	<0.01
	Negative	155	71	84	
Histology	Well diff.	120	59	61	<0.05
	Moderate diff.	106	63	43	
	Poorly and others	29	10	19	
Lymphatic invasion	Positive	200	100	100	0.35
	Negative	58	33	25	
Venous invasion	Positive	199	104	95	0.54
	Negative	59	28	31	
Liver metastasis	Positive	32	15	17	0.59
	Negative	226	117	109	
Tumor location	Right	86	42	44	0.6
	Left	172	90	82	

Table 2

**KIF4A mRNA expression in colorectal cancer tissues
using a realtime RT-PCR method**

	Relative ratio to β -actin (Median \pm SD)	<i>P</i> -value
Cancer tissue	3.55 \pm 8.67	0.002
Normal mucosa	2.07 \pm 5.01	